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Understanding the roles of *scalloped* and *vestigial* during wing development in *Drosophila melanogaster*

by

Ajay Srivastava

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

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Two roads diverged in a wood, and I - I took the one less traveled by, and that has made all the difference.

-Frost

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Understanding the roles of *scalloped* and *vestigial* during wing development in *Drosophila melanogaster*** submitted by **Ajay Srivastava** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Molecular Biology and Genetics**.

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Date: May 14, 2003

This thesis is dedicated to

Dadi, Mummy, Papa

and

Wendy

for their many personal sacrifices.

Abstract

Development of the Drosophila wing has proven to be an excellent model system that has contributed to our understanding of how growth and patterning occur in a secondary developmental field and also to our knowledge of how groups of cells differentiate to form an adult organ. Several genetic and molecular studies have demonstrated the importance of *scalloped* (*sd*) and *vestigial* (*vg*) during wing development and it is known that enhancer elements from the *vg* locus integrate the various signals into a wing specific effect. In this thesis I attempt to further understand the role these two genes play during wing development and morphogenesis.

While both SD and VG are nuclear proteins, only SD has a nuclear localization signal (NLS). Previous studies have shown that the presence of SD is critical for the nuclear localization of VG. Using an in-frame fusion between *vg* and the TEA domain of *sd*, I demonstrate that the NLS found within the SD TEA domain is likely responsible for the nuclear localization of VG. The fusion construct rescues wing phenotypes associated with *sd*, *vg* and the double mutants, thereby suggesting that for wing development ~30% of the SD protein is necessary and sufficient and in this respect the fusion construct mimics the native SD/VG complex. These data support the hypothesis that SD and VG function as a complex with SD providing the DNA binding ability and VG providing the activation function. Using the fusion construct, regulatory relationships between SD/VG, *wingless* (*wg*) and *senseless* (*sens*) are demonstrated and it is shown that *sd* function is likely needed for sensory organ development by *sens*.

Data from the molecular analysis of five recessive lethal alleles of *sd* show that while the mutation in one allele affects the TEA DNA binding domain, three others help define a VG binding domain in SD. It is known that for separation of the wing blade from the wing hinge, VG function is needed. Somatic clones expressing various truncations in the *vg* gene that remove the two previously identified activation domains demonstrate that for separation of wing hinge from the pouch, both activation domains must be present. Finally, an *in vivo* analysis of the SD interaction domain in VG is presented. It is demonstrated that the construct bearing the SID domain could be used to titrate SD from various tissues and thus this represents an important reagent that would help in further understanding the role *sd* plays in tissues other than the wing.

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Table of Contents

Chapter 1: General Introduction.....1

Chapter 2: A Vestigial:Scalloped TEA domain chimera rescues the wing phenotype of a *scalloped* mutation in *Drosophila melanogaster*.....29

Chapter 3: Further developmental roles of the Vestigial/Scalloped transcription complex during wing development in *Drosophila melanogaster*.....56

Chapter 4:Functional dissection of the TEA domain region found in a VG/SD TEA chimera during *Drosophila* wing development.....87

Chapter 5: Identification of a VESTIGIAL binding domain by molecular analysis of *scalloped* recessive lethal alleles in *Drosophila*.....105

Chapter 6: In vivo analysis of functional domains in VESTIGIAL.....140

Chapter 7: General Discussion and future directions.....158

List of Figures

Figure 1.1. Relative positions of the imaginal discs in third instar larvae.....25

Figure 1.2. Fate map of the wing imaginal disc and the corresponding structures in the adult *Drosophila* wing.....27

Figure 2.1. Construction of a fusion between VG and the SD TEA domain.....46

Figure 2.2. The *vg*::*sd*-TEA fusion construct is functional.....48

Figure 2.3. The TEA domain of SD is likely responsible for the nuclear localization of VG.....50

Figure 2.4. The *vg*::*sd*-TEA fusion construct is able to rescue a *sd* wing phenotype.....52

Figure 2.5. The fusion construct is able to induce expression of a downstream gene, *cut*.....54

Figure 3.1. Schematic of different regions of the wing disc and expression patterns of the Gal4 drivers used in this study visualized by monitoring expression of a UAS-GFP transgene.....73

Figure 3.2. Rescue of the D/V WG stripe by expression of the *vg*::*sd*TEA domain fusion.....75

Figure 3.3. The SD/VG complex promotes cellular proliferation.....77

Figure 3.4. Rescue of a *sd* mutation by exogenous WG.....79

Figure 3.5. Genetic interaction between *sens* and *sd*.....81

Figure 3.6. Expression of the *vg*::*sd*-TEA fusion construct restores SENS expression in wing discs derived from *sd* mutant animals.....83

Figure 3.7. A model depicting the relationships between WG, SD/VG and SENS during wing development.....85

Figure 4.1. Various constructs used in this study and features of the TEA domain.....99

Figure 4.2. Nuclear localization of VG in discs expressing various constructs.....101

Figure 4.3. Wing phenotypes associated with expression of full length *sd* and *sd* deletion constructs.....103

Figure 5.1. Characterization of *sd* lethal alleles and phenotypic classification.....128

Figure 5.2. The heterozygous wing-phenotype of recessive lethal and viable *sd* alleles.....130

Figure 5.3. Molecular lesions associated with the *sd* lethal alleles.....132

Figure 5.4. Evolutionary conservation of the residues affected by various *sd* lethal alleles.....134

Figure 5.5. VG localization in wing discs from various pupal lethal mutant allelic backgrounds.....136

Figure 5.6. A model to explain the molecular events that result in the variability associated with different *sd* alleles heterozygous with sd^{ETX4}.....138

Figure 6.1. Domain structure of VG.....148

Figure 6.2. Control Flp-out GFP clones in the wing disc.....150

Figure 6.3. Various UAS-*vg* deletion constructs used in this study.....152

Figure 6.4. Both activation domains in VG are required for adhesive properties of the cells in which VG is expressed and for the induction of *wg* in the hinge as judged by Flp-out clonal analysis.....154

Figure 6.5. *In-vivo* analysis of the Scalloped interaction domain (SID).....156

Chapter 1

General Introduction

Over the years *Drosophila* has proven to be an excellent model system for understanding basic developmental problems. Through the genetic screens of Nusslein-Volhard and Wieschaus (Nusslein-Volhard and Wieschaus, 1980) much has been learned about the genetic mechanisms underlying development. One of the biggest problems faced by an organism is the creation of cellular diversity starting from a single cell. It is now clear that this is achieved by turning on genes in a sequential manner (as evidenced by the segmentation of the Drosophila embryo) (Sanson, 2001). A second way of generating diversity is by making use of specialized cells that instruct their neighbours to initiate a particular developmental programme, thereby specifying the neighbors' identity (found during the development of the wing disc) (Irvine and Rauskolb, 2001). A general paradigm of development thus emerges, and involves progressive subdivision of an organism into smaller and smaller developmental units by the action of specific genes. It appears that each of the molecules at the top of the hierarchy, is either a transcription factor or a signaling molecule (Bondos and Tan, 2001; Lawrence and Morata, 1994).

The development of Drosophila follows the above mentioned developmental paradigm and begins with the deposition of an egg that is rich in maternal gene products organized in a gradient along the anterior-posterior axis (Ingham and Martinez Arias, 1992; St Johnston et al., 1989; St Johnston and Nusslein-Volhard, 1992). Thus, the unfertilized egg already has an axis polarized in the anterior-posterior co-ordinate that defines a primary embryonic field. In response to the gradient of maternal gene products a cascade of zygotic developmental program is later initiated that patterns the primary

embryonic field (Lawrence and Sampedro, 1993). The fertilized egg is patterned by the developmentally sequential activity of gap, pair rule, segment polarity and homeotic genes to give rise to an embryo that is made up of repeated segmental units. Each of these segmental units has an anterior (A) and posterior (P) as well as a dorsal (D) and ventral (V) polarity (Sanson, 2001). The polarity within a segment is specified by the action of a homeodomain containing gene *engrailed* (*en*) (along with *invected*) (Kornberg, 1981a; Kornberg, 1981b; Kornberg et al., 1985). Because the expression of *en* selects a group of cells to adopt a posterior identity, this gene is also referred to as a selector (Garcia-Bellido, 1975). The absence of *en* expression causes this group of cells to adopt an anterior identity (Lawrence and Struhl, 1982; Tabata et al., 1995). The embryo proceeds through development, including three larval instars, to form a pupa from which the adult emerges after about 10 days at 25°C (Demerec, 1950; Demerec, 1967). The adult bears little resemblance to the larva or pupa. Most of the adult head, thorax and appendage structures develop from specialized groups of cells called imaginal discs (Cohen et al., 1993; Simcox et al., 1991). Imaginal histoblasts (Simcox et al., 1991) give rise to the adult abdomen and other posterior structures while distinct imaginal rings are responsible for formation of salivary glands, gut and trachea (Hartenstein, 1993). The position of the imaginal discs and the imaginal histoblasts are shown in Figure 1.1 (Bryant and Levinson, 1985; Zalokar, 1947).

The development of external appendages has been the focus of studies into the secondary patterning mechanisms that give rise to a complex structure from groups of cells that have been assigned an identity based on their position in the developing embryo (Williams et al., 1993). The adult wing in Drosophila, due to its large size, complex

morphology and dispensable nature, has been of special interest because it presents the opportunity to identify genes that when perturbed result in a particular easily identifiable morphological defect.

Indeed, numerous genetic screens have identified genes involved in the development of the wing and understanding the function of these genes has elucidated the mechanisms responsible for patterning in a secondary field. In this introduction, the current understanding of wing development is reviewed. The role of *scalloped* (*sd*) and *vestigial* (*vg*) during this process is described, with special emphasis on the role of *sd*.

Early events during wing development

The development of the wing begins very early (at ~10hr germ band retracted embryo) when a group of about 30 founder cells is set aside as the wing disc primordium (Cohen et al., 1993). While the wing disc primordia are found in the second thoracic segment (T2), the haltere primordia on the other hand lie in the third thoracic segment (T3). Several homeotic genes of the *bithorax* and *antennapedia* complex limit the specification of the wing and haltere primordia to the T2 and T3 embryonic segments respectively (Bate and Arias, 1991; Carroll et al., 1995; Merrill et al., 1989; Merrill et al., 1987; Simcox et al., 1991; Vachon et al., 1992). Several methods have been employed to measure the number of cells in the wing and other disc primordia. The estimate of 30 cells in the wing disc primordia comes from the use of VG protein as a marker for these founder cells (Cohen et al., 1993; Williams et al., 1991). This estimate is in good agreement with the direct observations in the embryo reported earlier (Bate and Arias, 1991; Madhavan and Schneiderman, 1977). In *vg* null mutations, however, the wing and haltere discs are present suggesting that the VG expression is not the sole determinant of

the wing and haltere fate. It appears that the specification of the wing and haltere fate in the T2 and T3 segments is dependent upon the expression of zinc-finger proteins encoded by *escargot* (*esg*) and *snail* (*sna*) (Fuse et al., 1996).

The progenitors of wing discs and leg discs are found at the A/P mesothoracic segmental border in the embryo where the *wingless* (*wg*) and *decapentaplegic* (*dpp*) expression intersects (Cohen et al., 1993). These progenitors have an already established anterior posterior polarity marked by the posterior expression of *en* (Bate et al., 1993; Cohen et al., 1993; Lawrence and Struhl, 1996; Mann and Morata, 2000). Later on, the wing disc progenitor separates dorsally from the leg disc precursor and the A/P polarity is stably inherited through later stages of development in both the wing and the leg disc (Cohen, 1990; Klein, 2001). The dorsally located wing disc is marked by the expression of *vg* whereas the ventrally located leg disc is marked by the expression of *distalless* (*dll*) (Cohen et al., 1993).

In the wing disc the dorsal (D) ventral (V) subdivision occurs as soon as the first instar larva hatches (Klein, 2001) and is marked by expression of *apterous* (*ap*), a LIM homeodomain transcription factor (Blair, 1993; Blair et al., 1994; Diaz-Benjumea and Cohen, 1993). According to current understanding, the *ap* expression domain is established by the dorsal expression of *vein* (*vn*) which diffuses ventrally to form a gradient with high levels dorsally and low levels ventrally (Wang et al., 2000). This expression of *vn* in a gradient results in expansion of the *ap* expression domain ventrally which overlaps, (at the second larval instar when the wing field is established) with the ventral anterior expression of *wingless* (*wg*) (Ng et al., 1996). A high level of *vn* expression specifies the notum field and expression of *wg* marks the wing field. The high

expression of *vn* in the presumptive notum results in activation of the EGF-R pathway (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b) that activates the genes responsible for the formation of notum, like the genes of the Iroquois complex (Iro-C). The expression of *ap* divides the wing disc into two cell populations and the boundary of these two populations serves as the D/V organizer. Thus, the wing and the notum fields are specified by the actions of *wg* and *vn*, respectively. Through various larval instars the disc primordial cells are patterned, and in response to instructive signals grow in number to about 50,000 (late third instar, ~96h after egg-laying) (Bryant and Levinson, 1985; Klein, 2001). These epithelial cells appear as a monolayer in a sac called the wing disc that bears no resemblance to the adult wing. The monolayer of columnar epithelial cells in the wing disc is also continuous with the squamous cells of the peripodium (Gibson and Schubiger, 2001). The wing disc is designed and organized so that the proximal regions are peripheral in the disc and the distal regions lie towards the center of the disc. Thus, the problem of generating a three dimensional structure (wing) from a two-dimensional wing disc is overcome by the morphogenetic machinery. From outside to inside, the wing disc gives rise to the adult dorsal mesonotum, ventral pleura, hinge, and the wing pouch everts to form the wing blade (Bate et al., 1993; Klein, 2001). A fate map of the wing disc and the regions that form the corresponding adult structures is depicted in Figure 1.2 (Bryant, 1975; Campuzano and Modolell, 1992).

The wing disc is patterned in the anterior posterior (A/P) and the dorsal ventral (D/V) co-ordinates to subdivide the disc into specific regions with assigned positional identity. These specific regions in the disc have been shown to be regions of lineage

restrictions (Garcia-Bellido, 1975), or compartments (Blair, 2001; Crick and Lawrence, 1975). The boundaries (Irvine and Rauskolb, 2001) between these compartments serve as organizing centers or regions that have the ability to instruct the surrounding cells to adopt a particular positional value (Blair, 1995; Brook et al., 1996; Irvine and Rauskolb, 2001).

A/P Patterning

As mentioned earlier, the A/P subdivision of the wing disc occurs very early by expression of *en* in the posterior compartment. The *en* expressing P compartment cells express a short range secreted signaling molecule encoded by *hedgehog* (*hh*) (Lee et al., 1992; Tabata et al., 1992). While all of the posterior cells produce HH, only the cells immediately anterior to the posterior compartment are receptive to HH. The posterior compartment cells are rendered insensitive to HH by EN. A-compartment cells, farther from the A/P border are rendered insensitive to the effects of HH protein by the expression of a transcription factor Cubitus Interruptus (CI). In the A-compartment, CI protein is present in two forms, a full length form CI 155 and a truncated repressor form CI 75. The A-compartment cells farther away from the A/P border produce the repressor CI 75 which prevents the expression of HH responsive genes(Aza-Blanc et al., 1997; Methot and Basler, 1999). However, in the cells just immediately anterior to the A/P border the HH protein prevents the proteolysis of CI 155 and the accumulation of this form of the transcription factor results in expression of HH responsive genes (Aza-Blanc et al., 1997). The HH protein also alleviates the repressive effects of *Protein Kinase a C-1* and *patched* on *decapentaplegic* (*dpp*) expression in the cells just immediately anterior to the A/P border (Jiang and Struhl, 1995; Pan and Rubin, 1995). In response to HH

protein the cells anterior to the A/P border produce the long-range signaling molecule encoded by *dpp* (a BMP-4 homologue) (Basler and Struhl, 1994; Nellen et al., 1996; Zecca et al., 1995), that then patterns the wing in the anterior posterior co-ordinate (Brook et al., 1996; Lecuit et al., 1996; Nellen et al., 1996).

D/V Patterning

The dorsal ventral subdivision of the wing disc occurs during the first larval instar with the expression of *ap*, a LIM domain transcription factor, that defines the dorsal compartment (Diaz-Benjumea and Cohen, 1993). Later on (second and early third instar larval stage), the interaction between the dorsal and ventral cells results in establishment of a D/V organizer at the boundary between the dorsal and ventral compartment which then patterns the wing disc in the D/V axis (Diaz-Benjumea and Cohen, 1993; Williams et al., 1994). The interaction between D and V cells is mediated by the proteins encoded by *Delta* (*Dl*) and *Serrate* (*Ser*). Both DELTA (DL) and SERRATE (SER) act as short range signaling molecules that signal immediately across the D/V boundary. DL is expressed in the ventral compartment and is responsible for signaling from ventral to dorsal cells (Doherty et al., 1996; Klein and Arias, 1998b). SER, on the other hand, is expressed in the dorsal compartment and is responsible for signaling from dorsal to ventral cells (Couso et al., 1995; Kim et al., 1995). This signaling leads to the activation of receptor NOTCH (N) along the D/V compartment border (de Celis et al., 1996). It has been suggested that the ability of SER and DL to activate N is restricted to the D/V border by the glycosyltransferase (Bruckner et al., 2000; Moloney et al., 2000) activity of a protein encoded by *fringe* (*fng*) and expressed in the dorsal compartment (Irvine and Vogt, 1997; Irvine and Wieschaus, 1994; Kim et al., 1995). FNG makes the dorsal cells

more sensitive to signaling by DL from ventral cells (Panin et al., 1997). However, the dorsal cells are rendered insensitive to signaling by SER by the activity of FNG (Fleming et al., 1997). It has also been shown that N activation is limited to the D/V border, corresponding to the adult wing margin, by the effects of the POU-domain protein encoded by *nubbin* (*nub*) (Neumann and Cohen, 1998). Activation of N along the D/V border leads to expression of several margin-specific genes that are important for patterning of the wing pouch as well as in specification of wing margin elements. The *wingless* (*wg*), *vestigial* (*vg*) and *cut* (*ct*) genes are expressed on both sides of the D/V boundary in response to N activation (Irvine and Vogt, 1997; Neumann and Cohen, 1996; Rulifson and Blair, 1995). The WG protein is thought to act as a morphogen and is responsible for the expression of genes on both dorsal and ventral sides in a dose dependent manner, thus patterning the wing pouch for further development (Neumann and Cohen, 1997; Zecca et al., 1996).

Proximal distal patterning

The earliest subdivision of the wing disc in a proximal-distal axis occurs with the specification of the wing and notum fate in response to early *wg* expression and activation of the EGF-R pathway by *vn* (first larval instar to early second instar) (Klein, 2001). The expression of the Iro-C genes in response to the EGF-R pathway specifies the notum fate (Zecca and Struhl, 2002a; Zecca and Struhl, 2002b) and repression of *teashirt* (*tsh*) by *wg* and *dpp* specifies the wing proper fate (Wu and Cohen, 2002). The wing field is also marked by the expression of a transcription factor encoded by *nub* (Ng et al., 1995) that helps to limit N activation to the wing margin. Somatic clonal analysis of *nub* mutants suggests a role for this gene in proper development of the hinge and in growth of

the wing (Ng et al., 1995). Later development of the proximal wing hinge is under the control of *wg*, whereas the development of the distal wing pouch is under the control of both *wg* and *vestigial* (*vg*) (Klein and Arias, 1998a). The *wg* gene is expressed in a very interesting pattern in the third instar wing disc, with expression in a stripe corresponding to the future wing margin (Couso et al., 1994; Klein and Arias, 1998a; Williams et al., 1993) and then expression in two circles surrounding the wing pouch, an outer and an inner circle. The inner circle of *wg* expression is established in response to a signal from *vg* expressing cells of the wing pouch to the hinge cells, mediated by a hitherto unidentified signal (Liu et al., 2000; Rodriguez D del et al., 2002). This inner circle of *wg* expression is specified during early third instar and the outer circle is specified during late third instar. The peripheral expression of *wg* is responsible for the development of hinge structures in association with *homothorax* (*hth*), and *rotund* (*rn*). The expression of *wg* in the inner circle results in progressive subdivision of the hinge marked by different combinations of gene expression (Rodriguez D del et al., 2002).

***vestigial* and its role in signal integration during development of the wing disc**

Once the signaling from the D/V and A/P organisers is established, the integration of these signals into a wing specific effect, is directed by the wing pouch specific expression of *vg* (Klein and Arias, 1999; Williams et al., 1991). Thus, *vg* is downstream in the hierarchy of gene expression within the wing disc and is believed to control the wing specific fate of the cells in which it is expressed (de Celis, 1999). Consistent with this, ectopic expression of *vg* confers a wing like fate to tissues in which it is expressed (Kim et al., 1996). The expression of *vg* is spatially and temporally regulated by the enhancer elements found within the second and fourth introns of the *vg* transcription unit

(Kim et al., 1996; Williams et al., 1994). The intron-two enhancer element is also referred to as the boundary enhancer (BE) element and drives expression of *vg* along the D/V boundary (Williams et al., 1994). This enhancer element is active during the second larval instar and responds to signals from the N and WG signaling pathway, emanating at the D/V border and leading to the activation of this enhancer (Klein and Arias, 1998a). In accordance with its role in integrating N and WG signaling, the BE element has been demonstrated to have Su(H) binding sites (mediates N Signaling) (Kim et al., 1996) as well as TCF-1 binding sites (mediates WG signaling) (Klein and Arias, 1999).

The expression of *vg* throughout the wing pouch is controlled by another enhancer element located in intron four. This enhancer is referred to as the quadrant enhancer element (QE) for its ability to drive expression of *vg* throughout the four quadrants of the wing pouch (Kim et al., 1996; Klein and Arias, 1999). It is known that the presence of the BE element is required for the activity of this enhancer element (Kim et al., 1996). The QE element responds to N, WG and DPP signaling and is active during the late third larval instar. The N signal has two effects on this enhancer element. First, an autonomous negative effect on the activation of this enhancer at the D/V border is thought to work through an *E(spl)*-binding site found within the QE. Second, the ability of N signaling to activate this enhancer element non-cell autonomously through its role in maintenance of *vg* expression from the BE element is via activation of WG. This observation is supported by the reduced QE function in the presence of an ectopically expressed dominant negative WG molecule. It has been suggested that QE could respond to the WG signal through several TCF-1 binding sites found within this element. However, this response is dependent on the presence of VG itself (Klein and Arias, 1999). The presence of MAD

(Mothers Against Decapentaplegic) binding sites within this element makes it responsive to DPP signaling (Kim et al., 1997a). Together, the activity of these two enhancer elements leads to the stable expression of *vg* throughout the wing pouch (Kim et al., 1997b; Klein and Arias, 1999).

The *vg* gene encodes a 453 amino acid nuclear protein which has been implicated, both genetically and molecularly, in development of the wing (Halder et al., 1998; Simmonds et al., 1995; Simmonds et al., 1998; Williams et al., 1991). Recent studies have identified two functional domains *in vivo* (responsible for activation properties) (MacKay et al., 2003) as well as a domain that the VG protein needs to bind to the SD protein (Simmonds et al., 1998). Together these two proteins have been implicated as forming an essential transcription complex (Bray, 1999; de Celis, 1999; Halder et al., 1998). Mutations in *vg* affecting the BE element or the coding region have been identified and these result in loss of wing tissue. Clonal analysis of *vg* mutations in wing discs suggests that *vg* is important for growth and cell proliferation in an autonomous fashion (Liu et al., 2000; Simpson et al., 1981).

The *scalloped* locus

Genetics.

Mutations in the *sd* locus (*sd*:1-51.5/13F) were classically described as affecting the wing margin by producing notches and erosion of bristles (Grunberg, 1929). Most of the known wing mutations in *sd* were known to be viable. However, an EMS screen also identified several recessive lethal alleles and their characterization is presented in this thesis. The existence of lethal alleles implicates this gene in some vital function needed for the survival of the fly (Campbell et al., 1991). The phenotype associated with *sd* wing

mutations is quite variable and ranges from vestige-like wings (sd^{58}) to wings with just a few margin bristles missing (sd^{ETX4}). Somatic clonal analysis of these viable mutations affecting the wing has established that the requirement for *sd* function during wing development is cell autonomous (Halder et al., 1998; Liu et al., 2000). Mutations in sd^{UC1} are associated with extensive cell death during development of the wing disc and could be the reason for the notching in the wing associated with the viable alleles (James and Bryant, 1981). In addition to affecting the wings, *sd* mutations have also been associated with phenotypes in other tissues (Daniels et al., 1985). A temperature sensitive allele of *sd* is known to affect the legs, so that the legs appear twisted. Mutations that result in diminutive halteres have also been reported. Mutations resulting in ectopic bristle phenotypes are known, although the molecular mechanism behind this phenotype is yet to be determined (Campbell et al., 1991). Other mutant alleles of this locus also affect taste behaviour and muscle development (Inamdar et al., 1993).

Molecular Characterization.

The gene was cloned by transposon tagging, followed by a chromosome walk, and it appears that the *sd* transcription unit extends over 14 kb of genomic DNA. Most of the viable alleles affecting the wing map to the 5' end of the gene and appear to be mutations in regulatory elements. The *sd* locus has been demonstrated to produce several alternatively spliced transcripts and cDNAs corresponding to at least three of these transcripts exist (Campbell et al., 1991). Only one of these cDNA's is well characterized and it encodes a 440 amino acid protein containing an evolutionarily conserved TEA DNA (Burglin, 1991) binding domain within the N-terminal half (Campbell et al., 1992). The TEA domain has been shown to bind DNA in a sequence specific manner and has

been suggested to control the genetic regulatory network that is responsible for the development of the Drosophila wing (Guss et al., 2001). Genes encoding proteins that have a TEA DNA binding domain have been isolated from yeast to humans. The rescue of a *sd* wing mutation by the human homologue, TEF-1, demonstrates considerable functional conservation over the course of evolution (Deshpande et al., 1997).

Functional Analysis.

Enhancer trap assays as well as RNA *in situ* hybridization assays have demonstrated that the major transcript (E21) produced by *sd* is developmentally regulated in a spatio-temporal manner. Spatial and temporal expression levels in embryonic, larval and adult tissues have been determined. The peripheral nervous system, supraesophageal ganglion and the antenno-maxillary complex in the embryos display high levels of *sd* expression. In larvae, expression is detected in the optic lobe and cerebral hemispheres, as well as some cells of the ventral nerve cord. In the larval eye disc, the expression is localized to cells behind the morphogenetic furrow. Expression in the adult proboscis as well as the subesophageal ganglion has also been reported and correlates well with the reported role of this gene in taste behaviour (Campbell et al., 1992; Inamdar et al., 1993). However, by far the best studied tissue with respect to the role of *sd* is the wing disc. This tissue shows intense reporter gene expression from the enhancer trap in the wing pouch as well as in the notal areas.

Clonal analysis of both viable and lethal *sd* alleles during wing development shows that *sd* is needed for the viability of the cells (Halder et al., 1998; Liu et al., 2000). Also, clones deficient in *sd* result in lower levels of *wg* expression (Liu et al., 2000). Several studies on the regulation of *sd* during wing development (Varadarajan and

VijayRaghavan, 1999) have been carried out and it appears that *sd* is under the regulatory control of N signaling (Nagel et al., 2001). Genetically, *sd* has been shown to interact with the *vg* and *cut* (*ct*) loci. The TEA DNA binding domain from *sd* binds to the remote wing margin enhancer (Morcillo et al., 1996) from the *ct* locus (Jack and DeLotto, 1992). The role of *ct* in specification of the margin specific bristles is well documented and it is possible that *sd* plays a direct role in activation of this gene. Binding sites for *sd* have also been identified in numerous enhancers responsible for wing specific expression, like *spalt* (*sal*) and *Drosophila serum response factor* (*dsrf*) (Halder et al., 1998).

Using a variety of experimental approaches in this thesis, I try to further understand the role *sd* and *vg* play during wing development with special emphasis on the role of *sd*. The starting point for much of the work presented in this thesis came from the observations that *sd* and *vg* are genetically required for wing development, both may exhibit similar mutant wing phenotypes, and they have similar expression patterns in the wing discs. Also, the nuclear localization of the VG protein was known to be dependent upon the presence of SD, and SD and VG had been shown to bind to each other *in vitro* (Halder et al., 1998; Simmonds et al., 1998). Based upon these and other observations (included in chapter 2), it was hypothesized that SD and VG together acted as a transcription complex wherein SD provides the DNA binding ability and VG promotes the activation function. To test this hypothesis, *vg* was fused in frame to about 30% of the N-terminal encoding portion of *sd* that contains the TEA domain.

The following chapters deal with the creation of the fusion construct and the demonstration that this construct mimics the native transcription complex. Further, a putative TEA domain NLS responsible for the nuclear localization of VG is also

identified and characterized. Data from the molecular characterization of *sd* lethal alleles are also presented, and prove helpful in the *in vivo* identification of a VG binding domain in SD. Also, data demonstrating the role of the VG/SD complex in regulation of *wg* at the D/V boundary, as well as the role of *sd* in development of the sensory organ precursors by *senseless*, are also presented. Recent data have implicated *vg* in controlling the adhesive properties of the cells in the wing pouch and in inducing the hinge-specific expression of *wg* (Liu et al., 2000). The proposed differences in adhesive properties of cells within the pouch and hinge appear to play a role in separation of these structures during morphogenesis. Using Flp-out clones, the role of the two activation domains of VG on adhesive properties of the cells expressing it and on activation of hinge specific expression of *wg* is examined.

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Figure 1.1. Relative positions of the imaginal discs in a third instar larva.

A) A third instar larva showing the position of imaginal discs in relation to the segments. The segments are marked as T1-T3 for the three thoracic segments and A1-A8 for the abdominal segments. Dorsal structures are shown on the right and the ventral structures on the left. Genital discs corresponding to both male and female are shown in the same animal for the sake of convenience. The wing disc is highlighted by a shaded box and occupies T2-T3 segments. Modified from (Bryant and Levinson, 1985).

B) Lateral view of a larva showing the position of the wing imaginal disc (Zalokar, 1947).

(Images obtained from flybase at http://flybase.bio.indiana.edu/).

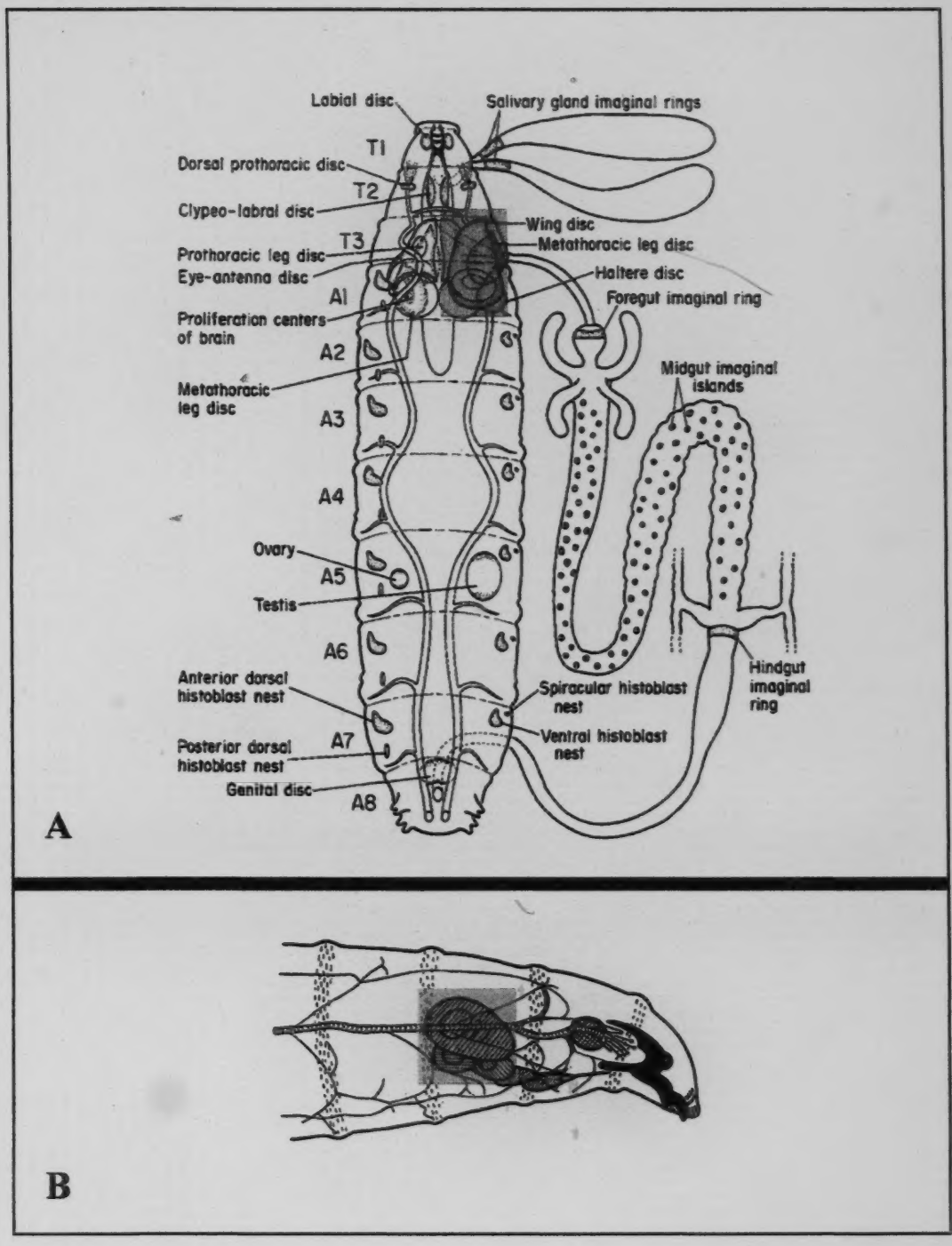
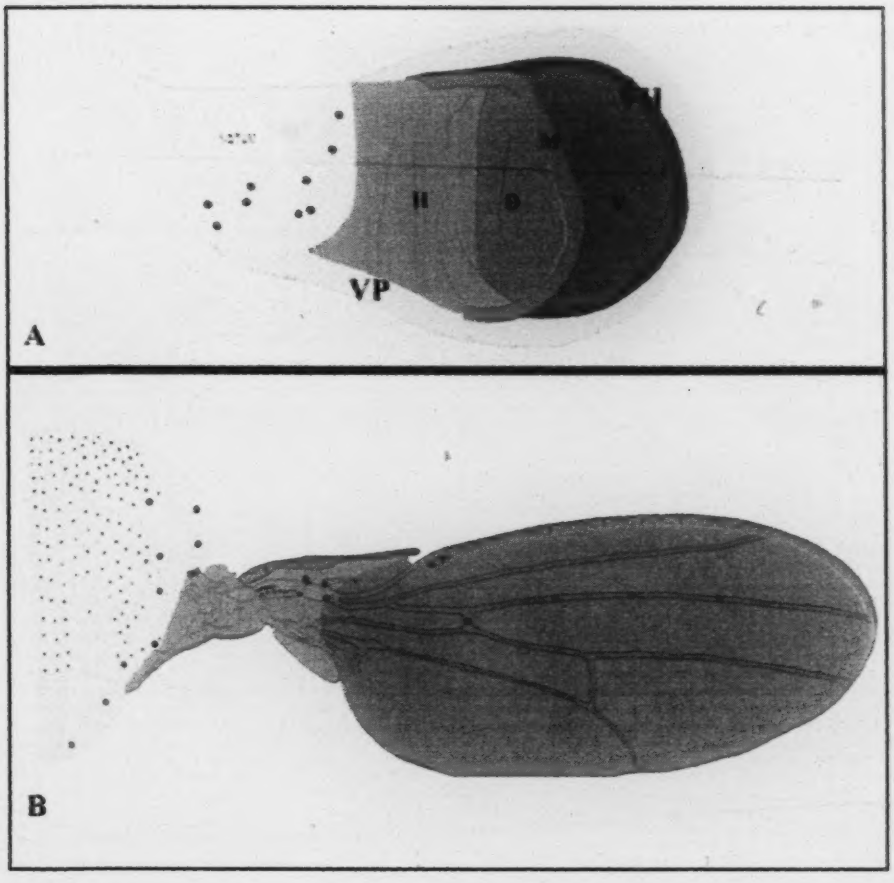

Labial disc
Salivary gland imaginal rings
T1
Dorsal prothoracic disc
T2
Clypeo-labral disc
Wing disc
T3
Metathoracic leg disc
Prothoracic leg disc
Eye-antenna disc
Haltere disc
A1
Foregut imaginal ring
Proliferation centers of brain
A2
Midgut imaginal islands
Metathoracic leg disc
A3
A4
Ovary
A5
Testis
A6
Hindgut imaginal ring
Anterior dorsal histoblast nest
Spiracular histoblast nest
A7
Ventral histoblast nest
Posterior dorsal histoblast nest
Genital disc
A8
A
B

Figure 1.2. Fate map of the wing imaginal disc and the corresponding structures in the adult *Drosophila* wing. Modified from a chapter by Stephen Cohen in The development of *Drosophila melanogaster* (Bate and Martinez Arias, 1993; Bryant, 1975). A) Fate map of a third instar wing imaginal disc is shown and the various regions fated to form the adult structures are colour coded. VP-Ventral Pleura, H -Hinge, D-Dorsal wing surface, M-Wing margin, V-Ventral wing surface, VH-Ventral hinge.

B) Schematic of an adult dorsal wing showing the notum (yellow), hinge (blue) and wing proper (green). The wing margin is coloured red and is decorated with margin bristles (Campuzano and Modolell, 1992).


VP
A
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Chapter 2

A VESTIGIAL:SCALLOPED TEA domain chimera rescues the wing phenotype of a *sd* mutation in *Drosophila melanogaster.* ¶

Introduction

The Drosophila wing provides an excellent model for studying developmental events in a secondary developmental field. In response to early primary patterning events in the embryo a group of founder cells is set aside which, in response to different patterning cues, forms a sac-like structure of epithelial cells which becomes the wing imaginal disc. The wing disc is divided into different compartments and the junctions between these compartments serve as organizers (reviewed in Brook et al., 1996). The various signals emanating from these organizing centers seem to be integrated and converted to a wing specific effect by the action of the VESTIGIAL (VG) protein, (Williams et al., 1994; Kim et al., 1996; Klein et al., 1998, 1999). This function of VG is mediated in partnership with a TEA domain-containing transcription factor (Burglin, 1991; Jacquemin et al., 1996) SCALLOPED (SD) (Simmonds et al., 1998; Halder et al., 1998; Guss et al., 2001). The expression of *vg* is controlled by two temporally distinct enhancer elements. One directs expression of *vg* along the Dorso-Ventral compartment boundary and is called the boundary enhancer element. The second enhancer drives expression in the four quadrants of the wing pouch and is called the quadrant enhancer.

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Co-author Julie MacKay did the micro-injection of the construct.

Together the *vg* enhancer elements control the expression of *vg* in the entire wing pouch (Kim et al. 1996.) The expression pattern of *sd* (Campbell et al., 1992) overlaps with the expression pattern of *vg* in the wing disc and mutations in the two genes give rise to similar wing phenotypes which are associated with the loss of wing margin bristles and wing tissue. In addition to its role in wing development, *sd* also has a vital developmental role in other tissues (Campbell et al., 1992).

During wing development, SD and VG have been shown to bind to each other (Simmonds et al., 1998; Halder et al., 1998; Paumard-Rigal et al., 1998). While a 56 amino acid domain in VG is responsible for binding SD (Simmonds et al., 1998), the portion of SD responsible for binding to VG was initially only localized to the carboxy terminal half of the protein (Simmonds et al., 1998). Recent studies have further localized this domain of SD to be within amino acid residues 220 to 341, separate from the DNA binding domain (Vaudin et al., 1999). This was based on homology with a domain found in the human homologue of *sd*, TEF-1, and the ability of TEF-1 to bind the human homologue of *vg*, Tondu (Simmonds et al., 1998; Vaudin et al., 1999). The TEF-1 gene is able to substitute for *sd* function during wing development (Deshpande et al., 1997). Similarly, Tondu has been shown to be able to partially substitute for *vg* function during wing development (Vaudin et al., 1999).

While both SD and VG are nuclear proteins (Campbell et al., 1992; Williams et al., 1991), only SD appears to have a putative Nuclear Localization Signal (NLS). Previous observations indicate that SD is required for the nuclear localization of VG (Simmonds et al., 1998; Halder et al., 1998) but the mechanism was not determined. Herein, a *vg*::*sd*-TEA domain fusion is used to show that the putative NLS found within the TEA domain

of SD is likely functional and utilized by VG to get into the nucleus by virtue of the ability of VG to bind to SD. In addition, other previous observations have established that VG and SD together act as a transcription complex wherein SD appears to provide the DNA binding ability and VG the activation function. (reviewed in de Celis, J.F. 1999; Bray, S. 1999; Mann et al., 2000; Guss et al., 2001; Halder and Carroll, 2001). The fusion construct is also used herein to conclusively show that, within the context of wing development, the SD-TEA motif is necessary and sufficient to provide the DNA binding ability and VG promotes the assembly of a wing specific transcription complex. This is because the fusion construct is able to promote wing formation in a *sd* mutant background as well as in a *vg* background and, more conclusively, in a doubly mutant *sd*;*vg* background. The fusion construct thus mimics the endogenous SD/VG heteromer in this respect and is also able to activate a known target of the native complex encoded by the *ct* gene. However, this fusion construct encodes only about 30% of the *sd* gene. Thus, the major portion of this vital gene is dispensable in the context of wing development.

Results

Construction of a functional *vg*::*sd*-TEA domain fusion.

SD and VG are both nuclear proteins (Campbell et al., 1992; Williams et al., 1991). Previous observations have demonstrated that the presence of SD is required for the nuclear localization of VG (Simmonds et al., 1998; Halder et al., 1998). In tissue culture, the expression of VG alone results in a predominantly cytoplasmic VG localization, whereas in the presence of a SD expressing plasmid, VG localizes to the nucleus (Halder et al., 1998). Also, ectopic expression of a VG protein deleted for the

SD binding domain results in cytoplasmic retention of VG (Simmonds et al., 1998). While the requirement of SD binding to VG for the nuclear localization of VG has been documented, it has not been demonstrated which portion of SD is responsible for this activity. Our analysis of the SD amino acid sequence suggests the presence of a putative bipartite nuclear localization signal (NLS) within the TEA domain of SD (Figure 2.1). Based on these observations, it was hypothesized that VG also could use this putative NLS to get into the nucleus. To test this hypothesis, a construct was made wherein the *vg* coding region was fused in frame with a PCR amplified DNA fragment corresponding to the TEA domain from *sd*, encoding SD residues 63-211 (Figure1). The fusion gene was cloned into pUAST (Brand and Perrimon, 1993) and injected into flies. Stable transformants were isolated and employed in experiments using the UAS-Gal4 system (Brand and Perrimon, 1993).

The functionality of the fusion construct was assessed in three ways. First, ectopic expression of *vg* results in the ectopic expression of a *sd* enhancer trap, sd^{ETX4} (Simmonds et al., 1998; Paumard-Rigal et al., 1998). This was used as a test to see if the fusion construct was able to induce *sd* expression under the control of a ptc-Gal4 driver (Figure 2.2 A,B,C). The results demonstrate that ectopic expression of this fusion construct occurs and results in ectopic expression of a *sd* enhancer trap, suggesting that the fusion construct retains VG function. Second, expression of this construct with a *vg*-Gal4 driver results in flies which display a new dominant phenotype involving defects in the bristles found on the thorax and notal areas as well as a cleft in the thorax (Figure 2.2 D,E). This phenotype is never seen when either *sd* or *vg* is expressed alone using a *vg*-Gal4 driver nor do we see it when UAS-*sd* and UAS-*vg* are co-expressed using a *vg*-Gal4

driver (data not shown). However, simultaneous expression of *vg* and *sd* is not the same as expression of just the fusion construct. It is well established that the fly is very responsive to subtle shifts in a delicate balance of SD and VG (Liu et al., 2000) with different outcomes associated with different levels of each protein. Moreover, the expression levels of the fusion construct versus the *vg* and *sd* constructs could also be influenced by the differing genomic positions of the respective transgenes. This suggests that the new phenotype is a dominant effect due to the expression of the fusion protein that possibly interferes with bristle patterning on the thorax and the notal area, again indicating that the fusion construct is functional. Finally, ectopic expression of the fusion construct with an *eyeless*-Gal4 driver produces ectopic wing tissue in the eye (Figure 2.2 F,G), in agreement with results when a full-length *vg* construct is ectopically expressed (Kim et al., 1996).

VG likely uses the NLS found within the TEA domain of SCALLOPED to get into the nucleus.

sd^{58} is a homozygous viable allele that produces an extreme wing phenotype which results from a very low level of *sd* expression (Simmonds et al., 1998; Campbell et al., 1991). VG is expressed in the wing pouch in wild type wing discs and is nuclear (Williams et al., 1991) (Figure 2.3 A-D; Z section in D). Wing discs from sd^{58} larvae stained using a rabbit anti-VG antibody show VG localized predominantly in the hinge and notal areas. Although the wing pouch is reduced in size, the low level of VG expression is diffuse but predominantly nuclear, suggesting that the low amount of SD in a sd^{58} background is complexed with VG (Figure 2.3 E-H; Z section in H). Over expressing the VG protein in the wing pouch of a sd^{58} mutant animal results in

predominantly cytoplasmic accumulation of VG, likely due to the non-availability of sufficient SD (Figure 2.3 I-L; Z section in L) to transport it into the nucleus. When sd^{58} male mutant wing discs that express the fusion construct (using a *vg*-Gal4 driver in a *vg* pattern) were stained with the VG antibody, the localization was clearly nuclear (Figure 2.3 M-P; Z section in P). This is consistent with the claim that VG translocates into the nucleus indirectly via the SD TEA domain, likely with the help of the NLS present within the TEA domain. However, a rigorous demonstration that this NLS is indeed sufficient to localize VG to the nucleus will require further subdivision and testing of the SD TEA domain.

Wing mutations associated with two *sd* alleles can be rescued by the *vg*::*sd*-TEA domain fusion.

Several lines of evidence suggest that both SD and VG are required for normal wing development (Simmonds et al., 1998; Halder et al., 1998; Varadarajan and VijayRaghavan, 1999; Guss et al., 2001). Firstly, expression of *vg* in tissues that already have a supply of SD results in ectopic outgrowth of wing tissue (Kim et al., 1996; Simmonds et al., 1998) as well as expression of downstream target genes like *sal* and *dsrf* (Halder et al., 1998). This ectopic outgrowth can be suppressed by taking away SD in a dose dependent manner (Simmonds et al., 1998). Secondly, targeted expression of SD alone, in tissues other than the wing, does not result in wing tissue outgrowth (Simmonds et al., 1998). It also fails to induce downstream target genes effectively (Halder et al., 1998). Thirdly, ectopic expression of *vg* deleted for the SD binding domain causes no ectopic wing tissue outgrowth and fails to induce *ct*, which is a downstream target gene (Simmonds et al. 1998). Finally, mutations of the SD-binding sites in the *vg* quadrant

enhancer drastically impair reporter gene activity (Guss et al., 2001). Thus, it is clear from these observations that the wing tissue outgrowth is a property that is conferred by the presence of VG, but this ability is contingent on a sufficient supply of SD. Based on the observations above, it was hypothesized that, within the context of wing development, SD plays the important role of allowing VG to gain access to target genes and bind to their enhancers. Once the SD/VG heteromer is bound to DNA via the TEA domain of SD, it is a property of VG that is responsible for activating downstream genes involved in wing development. If, indeed, the above is true and VG is responsible for the formation of a wing specific transcription complex, the fusion construct should be able to rescue *sd* wing mutations. Therefore, the fusion construct was expressed using a *vg*-Gal4 driver either in a sd^{ETX4} (Campbell et al., 1992) or a sd^{58} (Campbell at al., 1991) mutant background and tested to see if it was able to rescue the *sd* wing phenotype in F_1 males. The results demonstrate that the fusion construct is able to rescue the two mutant alleles almost completely. For the sd^{ETX4} allele (a weak hypomorph displaying notching of the wing) the rescue was virtually complete (Figure 2.4 A,C,D). There were occasional wing nicks seen and the fifth longitudinal vein (L5) was usually incompletely formed. In addition, bristle loss on the wing margin was also seen. The explanation for the loss of wing margin bristles and incomplete L5 could lie in the fact that the SD/VG heteromer is sensitive to the levels of SD and VG itself (Simmonds et al., 1998; Halder et al., 1998; Varadarajan and VijayRaghavan, 1999; Liu et al., 2000), so that over-expression of the fusion construct results in skewing of this delicate balance. The sd^{58} mutant displays a more extensive loss of wing tissue so the resulting wings appear very small (Figure 2.4 E). The sd^{58} allele is also associated with diminutive halteres (Vaudin et al. 1999).

Expression of the fusion construct in sd^{58} males using a *vg*-Gal4 driver results in a dramatic rescue of the wing as well as the haltere. The wing surface and the margin bristles are almost completely restored (Figure 2.4 F) and the size of the haltere is restored to wild type size (data not shown). However, the expression of UAS-*vg* alone driven by *vg*-Gal4 fails to rescue the sd^{58} wing phenotype (data not shown). As expected, the fusion protein will also rescue *vg* mutations since the fusion construct contains a complete *vg* gene (Figure 2.4 B). Finally, the UAS-*vg*::*sd*-TEA fusion will also rescue a *sd*;*vg* double mutant (Figure 2.4 H). The phenotype of unrescued vg^{BG}/vg^{79d5} sibs that did not receive the chromosome harboring the transgenic fusion gene is shown in Figure 2.4 G.

The *vg*::*sd*-TEA fusion mimics the native transcription complex and is capable of activating downstream target genes during wing development.

It has been shown that *sd* and *vg* bring about their effects by activating downstream target genes (Halder et al., 1998; Simmonds et al., 1998; Guss et al., 2001). Mutations in the *sd* gene have been shown to affect the expression of *ct* by inactivating the transcriptional enhancer for the wing margin in the *cut* locus, resulting in loss of mechanoreceptors and the bristles (Jack and DeLotto, 1992). The SD TEA domain has been shown to bind the wing margin enhancer in the *ct* locus (Morcillo et al., 1996). To see if the fusion construct was able to activate downstream genes, the expression of the CUT protein in *sd* mutant wing discs was examined. In sd^{58} male wing discs stained with a CUT antibody there is very little to no *ct* expression (Figure 2.5 A vs B). However, expression of the fusion construct with a *vg*-Gal4 driver in a sd^{58} male larva results in a

substantial restoration of *ct* expression (Figure 2.5 C) thus demonstrating that the chimeric fusion protein can mimic a native SD/VG heteromer in this respect.

Discussion

Taken together, it is clear that the fusion construct described herein mimics an endogenous SD/VG heteromer that is capable of activating downstream target genes responsible for differentiation of the wing tissue. Furthermore, the data support the hypothesis of a dual role for the native TEA domain within the context of wing development. The putative NLS found in the TEA domain is likely functional, and thus responsible for the nuclear localization of VG. Once inside the nucleus, the TEA domain confers on the fusion protein the ability to bind DNA elements near target genes and form a wing specific transcription complex capable of activating these downstream genes needed for the differentiation of bristles, vein tissue and inter-vein tissue. By analogy, this suggests that the SD/VG heteromer provides a context for a variety of signaling cues and initiates a variety of differentiation events required for wing formation. In this regard it acts as an integrator and trans*d*ucer of signals emanating from many signaling pathways responsible for formation of the entire wing. The results herein thus support a general paradigm for development in a complex multi-cellular organism where a transcription factor is expressed globally and then its activity is restricted locally within a particular tissue by means of a tissue-specific co-factor. These results also reinforce the notion put forth in Guss et al., (2001) that the SD/VG complex provides a qualitatively distinct function that is required for a wing-specific response to more global signaling cues.

It has been demonstrated previously that the SD protein could be divided into two domains, an N-terminal domain and a C-terminal domain. While the N-terminal contains the TEA DNA binding domain the C-terminal half contains a domain responsible for binding VG in-vitro (Simmonds et al., 1998). The ability of the fusion construct to work as a transcription complex clearly demonstrates that for the functioning of the VG/SD complex the TEA domain (amino acids 63-211) is essential and critical. In the context of wing development, the rest of the SD protein appears to be dispensable. The dispensability of such a large proportion of a vital transcription factor, at least in one specific tissue, will be of interest to those who study heterologous transcription factors and how they interact with other proteins. The fact that about 70% of the SD protein is not required for wing development should invite more serious studies of *sd* function in other tissues. *sd* is a vital gene and lethal alleles exist which map to the missing portion of the protein (Campbell et al., 1991; 1992). These lethal lesions must be concerned with these other *sd* functions; perhaps responsible for directing the ability of SD to interact with postulated other tissue-specific co-factors. The possibility that the residual SD activity remaining in the *sd* alleles used in the rescue experiment could positively influence the function of the fusion protein reported herein was also raised. While it is true that a leaky *sd* allele is used in the rescue experiments, it is because a viable fly is required to examine any wing phenotype. The remaining *sd* function does not even allow wing development in the presence of wild type levels of VG. Rather than assisting the fusion construct to function, this remaining SD will directly compete with the fusion protein for binding to natural targets and, in fact, would decrease the ability of the fusion

construct to rescue the *sd* mutations. The ability of the fusion protein to rescue the *sd* tester alleles is very strong despite this competition by the residual SD activity.

Recently, it has been proposed that binding of VG to SD switches the target selectivity of the complex (Halder and Carroll, 2001) either by VG globally affecting SD binding to DNA or by specifically enhancing binding of SD to the B-sites. For a detailed description of the A and B binding sites of SD see Halder and Carroll, (2001). It was also proposed that for this switch in target selectivity, regions outside of the SD Interaction Domain (SID) in VG are needed. While the model appears very compelling in the light of the in vitro experiments presented, it does not adequately explain some observations made by these authors and others. For example, how do *srf* sites, which have both A like and B like properties, get bound and activated by the VG/SD complex which has switched its target selectivity in favor of the B-sites (Halder and Carroll, 2001). The fusion construct reported herein rescues a *sd* wing phenotype and in the process must activate *srf*, which is responsible for the formation of inter-vein regions in the adult wing. It also induces *ct* expression, suggesting that in vivo the fusion construct activates enhancers having B-sites as in *ct* and A and B like sites as in the *srf* enhancer. If, indeed, VG binding was switching the target selectivity of SD in vivo in favor of B sites, then there would be problems associated with the fusion construct inducing genes having A and B like sites; or is it that the *srf* gene can be activated with SD alone. It was also suggested, that for the switch in target selectivity of SD, regions outside the SID (in VG) are required. However, Vaudin et al (1999) used Tondu to rescue a *vg* wing mutation and Tondu has no homology to VG outside of the SID. If regions outside the SID in VG were important for target selectivity, Tondu should not be able to rescue a *vg* wing mutation.

Yet a substantial level of rescue was demonstrated. Finally, if VG switches the target selectivity of SD by bringing about a conformational change in SD, then for this conformational change to be transmitted to the TEA domain, the C-terminal domain of SD would likely be needed as VG binds to this C-terminal region. The fusion construct reported herein only has a part of the N-terminal half (63-211) of SD that includes the TEA domain. The fact that the fusion construct is functional in the absence of the entire C-terminal half of SD clearly indicates that there is no critical role for this half of the protein in the context of wing development. Thus, it is likely that the modulation of TEA domain binding to DNA, in vivo, is effected by mechanisms in addition to the one proposed by Halder et al (2001). One such way could be by phosphorylating specific sites within the TEA domain. Currently we are testing this model by making specific mutations in the TEA domain in the fusion construct and then assaying for its ability to rescue a *sd* wing mutation.

The functionality of the fusion construct described herein also raises interesting evolutionary questions and possibilities with regards to this complex. If the two genes can function when fused together, then why are they separate? The answer could lie in one of the following possibilities. Firstly, separation of the two possibly gives the cell an extra level of control useful in regulating the final output from the complex. Secondly, separation of the two possibly allows the developing animal to utilize one of the components (in this case SD) in other developmental pathways by expressing it in many tissues and then limiting its function by means of tissue specific co-factors. As more DNA sequences from diverse organisms enter the database, it will be of interest to use the 'rosetta stone' approach (Marcotte et al., 1999) to investigate whether other orders of

insects, or more distant taxa, have sequences homologous to *vg* and *sd* in a single transcriptional unit. The results presented herein may also shed light on the workings of the human homologues of SD and VG, TEF1 and Tondu respectively. SD and TEF-1 are 98% identical within the TEA domain (Deshpande et al., 1997; Campbell et al., 1992). Also, the putative NLS of SD is conserved in its human counterpart TEF-1. Analysis of the protein sequence for Tondu reveals no NLS. It would be logical to extend the findings reported herein to humans and suggest that TEF-1 and Tondu together act as a transcription complex wherein Tondu gets into the nucleus with the help of the TEF-1 NLS and once inside the nucleus binds DNA via the TEA domain of TEF-1.

Materials and Methods

Transgene construction.

The transgene was made by PCR amplification of the *vg* coding region minus the stop codon. The DNA corresponding to the TEA domain of *sd* was also PCR amplified and fused in frame with the *vg* coding region. The downstream primer for the TEA domain contained the requisite stop codon. The transgene was cloned into pUAST and injected into yw flies using a Δ2-3 transposase source. For all amplifications, PFU polymerase from Stratagene Inc was used.

Stocks and Rescue experiments.

The transgene was expressed using the *vg*-Gal4 driver in all of the rescue experiments. The rescues were carried out at 25°C. sd^{ETX4} is described in detail in Anand et al., (1990) and sd^{58} is described in Campbell et al., (1991). *vg*-Gal4, constructed by Morimura and Hoffman (unpublished) and obtained from Sean Carroll, directs expression under control of the *vg* boundary enhancer. This enhancer drives expression at the dorso-

ventral boundary and the hinge and notal areas of the wing disc. The *sd*;*vg* double rescue experiment was carried out by examining the male offspring of a cross between sd^{ETX4}; vg^{BG} *vg*Gal4; + homozygous females and w; vg^{79d5}; UAS*vg*::*sd*TEA/+ males. From such a cross, all the male progeny are sd^{ETX4}; vg^{BG} *vgGal4*/vg^{79d5} and half of these males inherit the fusion construct gene on chromosome III and are rescued, while the other half do not inherit the fusion construct and are not rescued. Furthermore, half of the female progeny will also be rescued for *vg* since they are all heterozygous for the two *vg* alleles in the cross and half of the females will also inherit the fusion construct gene. These females are not doubly rescued, however, since they are not homozygous for the sd^{ETX4} allele.

Immunohistochemistry.

All immunohistochemical staining was done as described in reference Williams et al. 1991. A rabbit anti-VG antibody was used for localizing VG and a 2B10 antibody (from Developmental Studies Hybridoma Bank, University of Iowa) was used for staining the Cut protein. The secondary antibodies were a Cy3 conjugated anti Rabbit Ab for localizing VG and a Cy3 conjugated anti mouse Ab for localizing Cut (Jackson ImmunoResearch). The nuclei were stained using Hoechst 33258 (Molecular Probes).

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Figure 2.1. Construction of a fusion between *vg* and the *sd* TEA domain. A) The coding region for *vg* was PCR amplified without the stop codon and ligated to a PCR amplified TEA domain from *sd*, which had an engineered stop codon at the 3' end. The resulting fusion was cloned into pUAST and injected into *yw* flies. B) The amino acid sequence of the region of SD (containing the TEA domain) found in the fusion construct is shown. The TEA domain-containing region spans amino acids 63-211 of the SD protein (Hwang et al., 1993). The nuclear localization signal, as deduced by using profile search software from Expasy (http://www.expasy.ch/), is boxed.

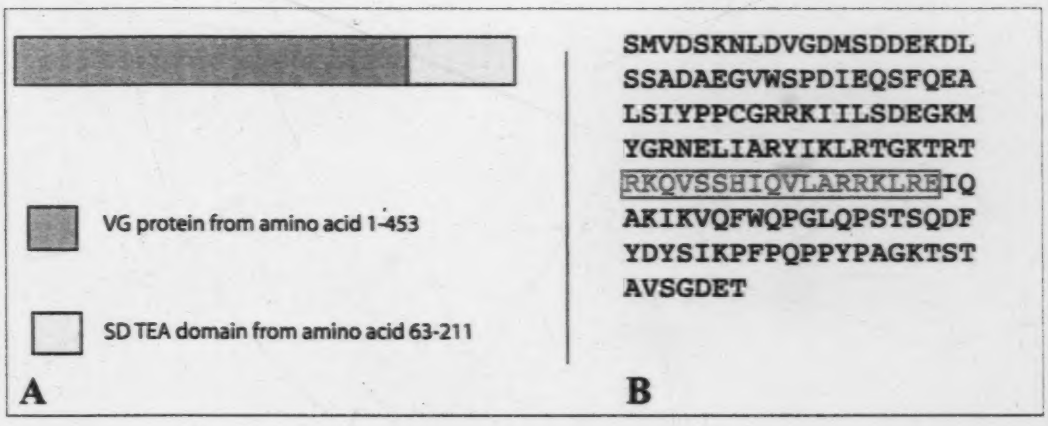
VG protein from amino acid 1-453
SD TEA domain from amino acid 63-211
A
SMVDSKNLDVGDMSDDEKDL
SSADAEGVWSPDIEQSFQEA
LSIYPPCGRRKIILSDEGKM
YGRNELIARYIKLRTGKTRT
RKQVSSHIQVLARRKLREIQ
AKIKVQFWQPGLQPSTSQDF
YDYSIKPFPQPPYPAGKTST
AVSGDET
B

Figure 2.2. The *vg::sd-TEA* fusion construct is functional. A-C Wing discs stained for β-galactosidase (lac-Z) activity. D-E) Scanning electron micrographs of the thorax from adult flies. A) Lac-Z staining of a wing disc derived from a sd^{ETX4} male larva showing the endogenous domain of *sd* expression that overlaps the wing pouch and the hinge areas. B) Wing disc stained for lac-Z activity showing the domain of expression of UAS-lacZ driven by a *ptc*-Gal4 driver. Notice the expression along the antero-posterior border. C) Ectopic expression of the UAS-*vg*::*sd*-TEA fusion construct with a *ptc*-Gal4 driver results in the expression of sd^{ETX4} in the wing disc along the antero-posterior border showing that the fusion construct is functional. D) Thorax of an adult wild type fly showing the symmetrically arranged bristles. E) Thorax from a fly in which the fusion construct has been expressed using a *vg*-Gal4 driver. Notice the mis-arrangement of the thoracic bristles as well as the cleft in the thorax and scutellum. Notice also the wing like tissue outgrowth from the scutellum. F) Ectopic wing tissue in the eye using an *eyeless*Gal4 driver to express the *vg::sd-TEA* fusion protein. G) same as F, enlarged to show bristle detail.

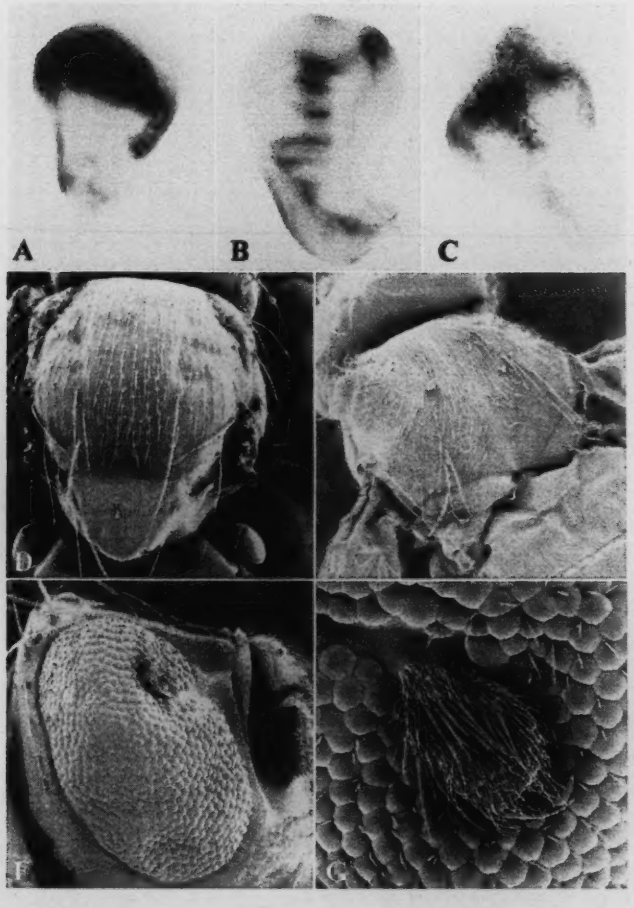
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Figure 2.3. The TEA domain of SD is likely responsible for the nuclear localization of VG. A-P, Confocal scans of wing discs from male late third instar larvae, A,E,I,M stained with a rabbit anti-VG antibody to visualize the VG protein (red channel) and B,F,J,N stained with Hoechst 33258 (blue channel) to visualize the nuclei. The overlap between the two channels is presented in panels C,G,K,O. Panels D,H,L,P are optical Z sections of the relevant genotype. A-D) wild type wing-discs showing the pattern of VG expression (red channel) Panel B shows the nuclei (blue channel) and panel C is the overlap between the two. The VG protein is clearly localized to the nucleus as ascertained by the punctate nature of VG protein and the magenta colour produced by the overlap of the red and blue channels. D, shows the nuclear localized VG in a Z section. E-H) Wing discs from sd^{58} male larvae showing the expression of VG in a smaller domain (compare to A). The VG protein is predominantly localized to the nucleus as ascertained by the magenta colour in panel G and in the Z section shown in panel H, suggesting that the low level of SD in sd^{58} is bound to endogenous VG. I-L) Wing discs over expressing VG using a *vg*-Gal4 driver in a sd^{58} genetic background result in the cytoplasmic localization of VG, clearly evident in panel I, panel K and the Z section in L. M-P) Over expression of the fusion construct by a *vg*-Gal4 driver in a sd^{58} genetic background results in a nuclear localization of VG. Notice the punctate nature of the staining in M, and the overlap in O producing the magenta colour. Also compare the Z sections in panels L and P.

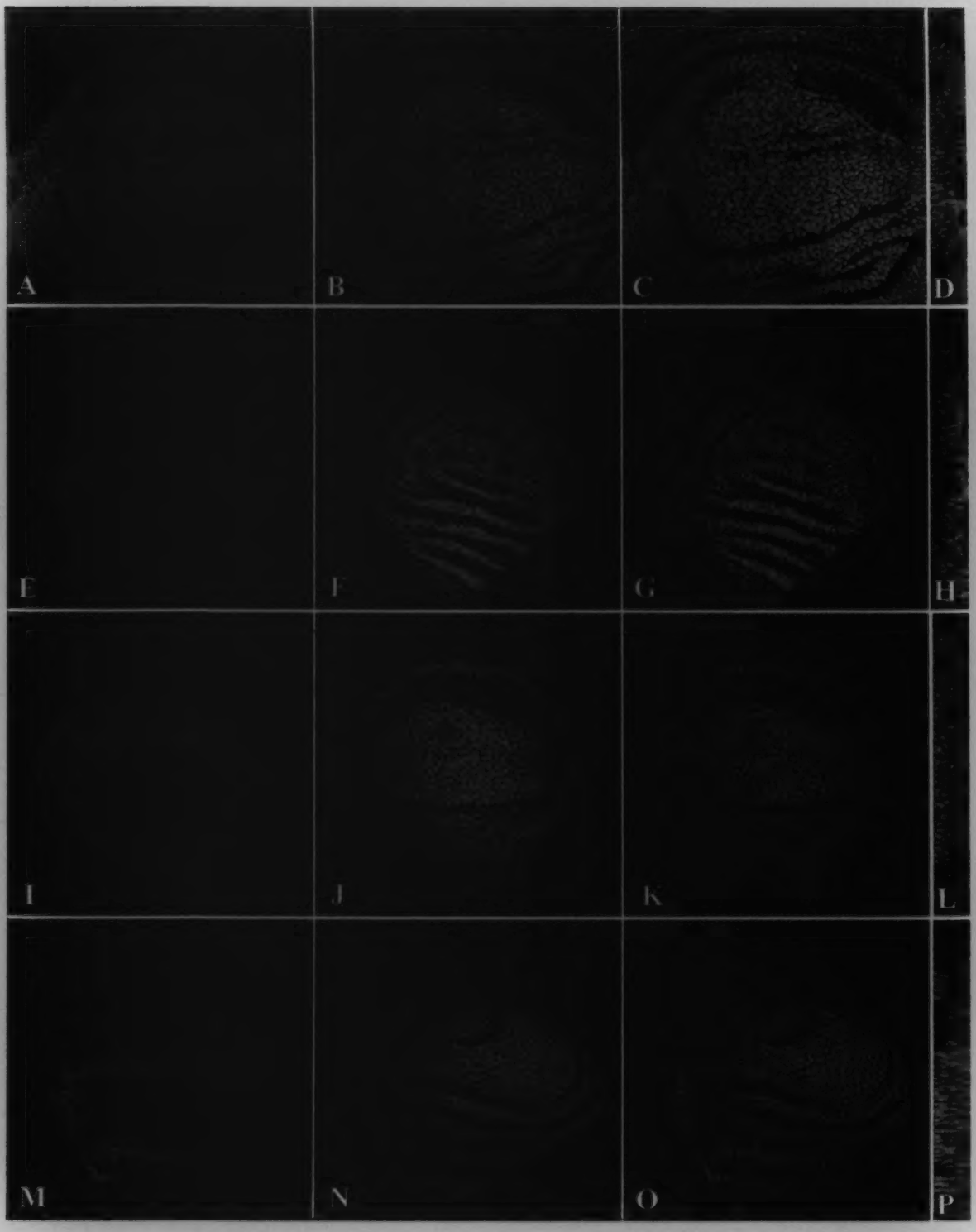
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Figure 2.4. The *vg::sd-TEA* fusion construct is able to rescue a scalloped wing phenotype. A-H, Whole mounts of wings from adult flies. A) Wing from a wild type male fly showing the uniform margin with all the bristles intact. B) Rescue of a *vg* mutation with the fusion gene. This panel shows a wing from a vg^{79d5}/vg^{BG} female also carrying a *vg*-Gal4 driver and the UAS-*vg*::*sd*-TEA gene. C) Male wing from sd^{ETX4} fly showing the notching of the wing and the loss of bristles. D) Expression of the fusion construct using the *vg*-Gal4 driver results in a complete rescue of the sd^{ETX4} wing phenotype that is fully penetrant, in that all F_1 males exhibit the rescue phenotype. For the rescue assays, either homozygous sd^{ETX4} or sd^{58}; *vg*-Gal4 females were crossed to homozygous UAS-*vg*::*sd*-TEA males, and F_1 males were examined for the rescue phenotype. Note the restoration of wing shape and the bristles on the margin. E) Wing from a male sd^{58} fly showing the loss of wing tissue and bristles. F) Expression of the fusion construct using a *vg*-Gal4 driver in a sd^{58} male results in the rescue of the sd^{58} phenotype. Again, all F_1 males exhibited this level of phenotypic rescue. Note the restoration of wing tissue as well as the margin bristles. G) Wing from an unrescued *sd; vg* male double mutant of genotype sd^{ETX4}, vg^{79d5}*vgGal4*/vg^{BG}. H) Wing from a rescued double mutant male of genotype sd^{ET}; vg^{79d5}/vg^{BG} *vg*-Gal4; UAS-*vg*::*sd*-TEA/+ . The wings in panels B,G, and H are all of relevant siblings from the same vial. See Methods for the complete genotypes of the parents in this cross.

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Figure 2.5. The fusion construct is able to induce expression of a downstream gene, *ct*. Wing discs stained for the CUT protein (red) using antibody 2b10. A) Wing disc from a wild type male larva showing CUT expression along the dorso-ventral compartment boundary. B) Little or no CUT expression is seen in wing discs from a sd^{58} male larva. C) Expression of the fusion construct using a *vg*-Gal4 driver in a sd^{58} male restores the expression of CUT along the dorso-ventral boundary.

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Chapter 3

Further developmental roles of the VG/SD transcription complex during wing development in *Drosophila melanogaster.* ¶

Introduction

The wings in Drosophila develop from a sac made up of a single layer of columnar epithelial cells called the wing imaginal disc that gives rise to the hinge, blade and also the mesonotum of the adult (reviewed in Cohen, 1993). However, recent evidence also points to the existence of signaling between peripodial cells and the columnar epithelial cells during wing development (Gibson and Schubiger, 2000). The adult wing in Drosophila is made up of the wing hinge and wing blade (Figure 3.1 A). Apart from its function in flight, the wing blade also serves a sensory function and is covered with sensory bristles along the anterior margin. During wing development, several genetic pathways interact to partition these structures. The wing disc is determined very early in development with the specification of a group of embryonic cells to form the wing disc progenitors (Cohen, 1990; Cohen, 1993). Subsequently, through the larval instars, these cells proliferate to form the wing disc which contains ~53000 to 75000 cells (Klein, 2001; Whittle, 1990). As the wing disc grows in size, it is progressively patterned into smaller sub-divisions that can be inferred by the differential expression of genes within each sub-division.

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As a result, the wing disc is divided into four compartments: anterior, posterior, dorsal and ventral (Crick and Lawrence, 1975; Garcia-Bellido et al., 1973).
The borders between these compartments are thought to act as organizing centers, to produce a signaling source that brings about initial patterning of the wing (Basler and Struhl, 1994; Diaz-Benjumea and Cohen, 1993).

The cells of the wing disc respond very early to the expression of *wg* and EGFR signaling pathways, which divide the disc into the structures required for flight and the part that forms the body structure called the notum (Klein, 2001). The wing disc is patterned along the anterior/posterior (A/P) axis by the combined action of *hh*, *en*, and *dpp* (Brook et al., 1996). The patterning along the D/V axis is effected by the actions of *ap*, and by *Ser* and *fng* (Brook et al., 1996; Irvine and Vogt, 1997; Mann and Morata, 2000). Together, these proteins lead to activation of the *N* signaling pathway at the D/V compartment border. This ultimately produces localized secretion of the WG morphogen along the D/V border in mid to late third instar, acting as a long range signaling molecule to pattern the adult wing (Neumann and Cohen, 1997a; Zecca et al., 1996). In addition to being expressed at the D/V border, corresponding to the presumptive wing margin, *wg* is also expressed in three rings in the proximal regions of the wing disc. This expression surrounds the wing pouch and, in combination with other molecules, specifies the hinge fate (Couso et al., 1994; Klein and Arias, 1998). Both *vg* and *sd* are also under the control of the *N* signaling pathway (Couso et al., 1995; Kim et al., 1996; Nagel et al., 2001; Williams et al., 1994).

VG is a nuclear protein that has been shown to be responsible for conferring a wing like fate to cells in which it is expressed (Williams et al., 1991). Functional analysis

of VG has identified two necessary domains *in vitro* (Halder et al., 1998; Vaudin et al., 1999) and *in vivo* (MacKay et al., 2003) and a SD interaction domain (Simmonds et al., 1998). VG functions together with SD, a co-factor belonging to the TEA/ABAA domain-containing family of transcription factors (Burglin, 1991). The genetic requirement of these two genes in establishing wing fate is well documented (Campbell et al., 1992; Campbell et al., 1991; Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002; Vaudin et al., 1999). Within the wing field, SD/VG has been shown to activate cis-regulatory elements of some of the target genes directly, which in combination with other signaling pathways are responsible for wing development (Guss et al., 2001). Clonal analysis of *sd* mutations has shown that homozygous *sd* mutant cells are deficient in the expression of *wg* (Liu et al., 2000). It has also been proposed, using mis-expression studies, that *sd* and *wg* operate in an auto-regulatory loop with *vg* (Varadarajan and VijayRaghavan, 1999). The expression of *vg* and *sd* appears to be activated as a gradient, with highest protein levels seen in cells along the D/V border (Campbell et al., 1992; Williams et al., 1993). They are thought to be under the control of enhancer elements that respond to signals emanating from the compartment borders (Campbell et al., 1992; Deshpande et al., 1997; Kim et al., 1997; Kim et al., 1996).

The anterior of the adult wing margin in Drosophila is decorated with sensory bristles that are formed by the action of proneural genes such as the *achaete scute* complex (Modolell, 1997). The specification of the sensory bristles occurs by the selective accumulation of proneural proteins of the *achaete scute* complex, in a particular cell called the sensory organ precursor (SOP). This accumulation of proneural proteins in

the SOP cells distinguishes it from the neighboring ectodermal cells in which the accumulation of the proneural proteins is repressed by the action of the *N* pathway (Nolo et al., 2000). The role of *ct* (Blochlinger et al., 1990; Blochlinger et al., 1991) and *sd* (Campbell et al., 1992; Campbell et al., 1991) and their relationship in the development of the margin bristles is established (Morcillo et al., 1996; Simmonds et al., 1998; Srivastava et al., 2002). The role of a proneural gene *senseless(sens)* in the formation of sensory bristles has also been documented (Nolo et al., 2000). However, the relationship between *sd* and *sens* in this process has not yet been described.

Herein, the roles of *sd* and *vg* during important events necessary for wing development and morphogenesis are examined by utilizing a *vg::sd*-TEA fusion that replicates the function of the SD/VG complex (Chapter 2; Srivastava et al.,2002). The relationship of the SD/VG complex to the appearance of the D/V stripe of *wg* expression is clarified. It is shown that the SD/VG complex can restore the *wg* D/V expression stripe in a *sd* mutant background. In this respect, SD/VG either induces or maintains the expression of *wg* at the margin. Also, we show that exogenous *wg* expression appears to be able to partially rescue *sd* mutations. The role that *sd* plays during bristle specification by *sens* is also examined and it is demonstrated that *sd* likely has a role in proper *sens* expression for SOP development at the wing margin. Thus, this study provides further insights into the role of SD/VG complex during wing development by utilizing a *vg::sd*-TEA fusion. A likely molecular basis for the rescue of *sd* mutations by this fusion *vis a vis* the role the complex has in maintaining or inducing a portion of the *wg* expression pattern is also provided.

Results and Discussion

The VG/SD transcription complex restores the *wg* dorso/ventral-specific expression in *sd* mutant wing discs and promotes proliferation.

In chapter 2, it is demonstrated that a fusion between *vg* and the TEA domain of *sd* encodes a protein that functions like the native SD/VG transcription complex in the context of wing formation. This fusion can rescue *sd* or *vg* single mutations as well as the double mutant wing phenotype (Srivastava et al., 2002). Several lines of evidence suggest that this rescue could be happening via the *wg* pathway. Firstly, clonal analysis of *sd* mutations has shown that wing discs are deficient in specific aspects of *wg* expression (Liu et al., 2000). Secondly, it has been proposed that *sd* can induce *wg* expression (Varadarajan and VijayRaghavan, 1999) and might be involved in the maintenance of the *wg* D/V specific expression (Liu et al., 2000). Thirdly, the role of *wg* in growth and proliferation of the cells in the wing disc is well documented (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1997b; Phillips and Whittle, 1993; Zecca et al., 1996). Finally, transdetermination of leg disc cells to wing like cells is achieved by over expression of *wg* in the leg disc (Maves and Schubiger, 1995). Thus, it is likely that the rescue of the *sd* wing phenotype by the fusion construct (Srivastava et al., 2002) is mediated by *wg*.

To test this hypothesis, the localization of WG was examined by staining wing discs from wild-type, *sd*, and *sd* transformants expressing the *vg::sd*-TEA fusion construct using a *vg*-Gal4 driver. The different regions of the wing disc are depicted in a schematic of the wing disc (Figure 3.1 A). The expression patterns of the *vg*-Gal4 driver, as well as others used in this study, were visualized using an UAS-GFP transgene and are

shown in Figure 3.1 B-D. The localization of WG was visualized by using an anti-WG antibody (see Material and Methods). Simultaneously, these discs were also examined for VG localization as a marker for normal versus mutant wing discs, and also as a marker for the spatial expression of the fusion construct. The normal spatial relationship between WG and VG expression is shown in Figure 3.2 A-C. In wild-type wing discs, WG is localized in a typical distinct pattern in the hinge and at the boundary between the dorsal and ventral compartments, which forms the wing margin in the adult wing. VG, on the other hand, is present throughout the wing pouch with higher levels at the D/V compartment border (Figure 3.2 A and C). In *sd* mutant wing discs, the D/V boundary-specific localization of WG is lost, while the hinge-specific localization appears to be normal (Figure 3.2 E,F). The domain of VG localization is also smaller (Figure 3.2 D) but occupies the space where the D/V boundary-specific WG would be (compare Figure 3.2 C and F). However, expression of the fusion construct in *sd* mutant wing discs restores the D/V boundary-specific localization of WG (Figure 3.2 G,H,I). It is likely that this restoration of WG D/V expression is responsible for the previously described rescue of *sd* mutant wings (Srivastava et al., 2002). To ascertain if this rescue happens by promoting cellular proliferation, the level of mitotically active cells was determined using an anti-PH3 antibody as a marker for mitotic cells (Hendzel et al., 1997). Staining of wing discs from wild type larvae shows the presence of numerous mitotically active cells (Figure 3.3 A). Wing discs derived from sd^{58} mutant larvae show a lower number of mitotically active cells (Figure 3.3 B) as well as a smaller wing pouch. However, discs derived from sd^{58} larvae expressing the fusion construct appear to restore the frequency of mitotically dividing cells to wild type numbers (Figure 3.3 C). Thus, one can infer that

the rescue of *sd* mutants by the fusion construct is the result of its ability to restore WG expression along the D/V border as well as promote cellular proliferation within the wing pouch. This is further supported by the rescue of the sd^{58} wing phenotype by the fusion construct expressed in a sd^{58} background, as previously reported (Srivastava et al., 2002).

Rescue of a *sd* wing phenotype by exogenous WG.

As demonstrated above, restoration of the WG D/V pattern of expression by the fusion construct could explain the rescue of a *sd* wing phenotype. Two lines of evidence support this notion. Firstly, if this restoration of the WG D/V expression stripe is responsible for the rescue, then exogenous expression of *wg* should also be able to rescue a s*d* mutant wing phenotype. To test this, a UAS-*wg* transgene was expressed under the control of a *vg*-Gal4 driver in *sd* mutant backgrounds, sd^{58} and sd^{ETX4} (Figure 3.4 C-F). The *vg*-Gal4 driver directs expression under the influence of the *vg* boundary enhancer element along the D/V compartment border in the wing disc (Morimura and Hoffmann, unpublished observations, and Figure 3.1 C) and, as such, is a relatively weak driver. However, expression of the UAS-*wg* construct in sd^{58} mutant wing discs results in a partial rescue of the *sd* wing phenotype which appears to be confined to the proximal wing blade (compare figure 3.4 A with 3.4 E and with 3.4 F). Because the partial rescue of the wing phenotype could be caused by this transgenic combination, the relative amount of protein produced by the UAS-*wg* transgene at the D/V boundary was also examined. The data suggest that the level of rescue of the *sd* phenotype is consistent with the amount of exogenous WG at the D/V boundary, since very low levels of the WG protein are observed compared to what is found in a wild-type disc (results not shown). The highest level of WG protein is confined to the proximal regions of the wing pouch.

which correlates well with the wing rescue data. It is also important to point out that the sd^{58} wing phenotype is not variable in different genetic backgrounds, and hence it is inferred that the partial rescue observed is due to the expression of *wg* from the transgene. sd^{ETX4} is a weak allele of *sd* and displays a range of wing phenotypes. Varadarajan and VijayRaghavan (1999) employed an arbitrary scale from + to –5 to measure this variability, where + refers to wild type wings and – scores refer to progressively severe notching of the wings (Fig 2A in Vardarajan and VijayRagnavan, 1999). According to that report, a majority of the sd^{ETX4} wings had a score of -3 or more severe. To further support the claim that exogenous *wg* rescues *sd* mutations, rescue of sd^{ETX4} by exogenous expression of *wg* was also examined (Figure 3.4 C and D). It is clear from the data that the wing rescue in this mutant background is almost complete and is seen in 100% of the flies. The wings are restored to a score of –1 to –1.5 on the arbitrary scale. While both *sd* mutants were rescued in terms of the wing blade, rescue of the margin bristles by exogenous *wg* was not observed. Thus, it is likely that *wg* also needs other functions for formation of the margin bristles.

The second line of evidence utilizes a UAS-$dTCF^{DN}$ construct. dTCF acts within the nucleus as a transducer of *wg* signal and a UAS-$dTCF^{DN}$ construct expresses a dominant negative (DN) form of the dTCF protein, which results in a compromised WG signal (Cadigan, et al., 1998). Thus, if the D/V specific expression of *wg* was responsible for the rescue of the *sd* mutant phenotype, the expression of the DN form of dTCF should be deficient in the D/V WG signal and hence produce a phenotype similar to *sd* mutant wings. The C96-Gal4 driver expresses a UAS transgene at high levels along the D/V border (Figure 3.1B). In addition, the C96-Gal4 driver also causes expression in other

tissues (Gustafson and Boulianne, 1996). Thus, expression of the UAS-*dTCF*^{DN} construct with the C96-Gal4 driver results in reduced viability of the progeny because of expression of dTCF in tissues other than the wings. However, surviving flies from this cross do produce a *sd*-like phenotype in the wing (compare Figure 3.4 B and C), strengthening the notion that the rescue of the *sd* wing phenotype is likely via *wg* expression at the D/V compartment border. A similar phenotype is observed when the construct is expressed using a *vg*-Gal4 driver (data not shown). So, a *sd* mutant can be rescued by *wg* expression at the D/V boundary and a compromised WG signal at the D/V border results in a *sd*-like wing phenotype. Thus, it appears that, within the narrow context of D/V boundary-specific expression of *wg*, *sd* acts upstream of *wg* in the genetic hierarchy for wing development, or it simply emphasizes that there is a feed-back loop involving these genes. This result is in accord with that of Liu et al., (2000) in that *sd* may be required for the expression or maintenance of *wg* at the D/V boundary.

Genetic interaction between *sd* and *sens*.

When the *sd* gene is mutated, the phenotype includes not only the wing margins but also the sensory organs that are found at the wing margins. In addition to the loss of wing margin bristles, there is also a reduction in the number of cells, which results in notching of the wings (Compare Figure 3.4 A vs 3.5 A). This reduction in the number of cells is thought to be a result of apoptosis (James and Bryant, 1981). In addition, over expression of *sd* is also associated with apoptotic cell death (Liu et al., 2000). *Lyra (Ly)* mutations, on the other hand, result in the loss of the anterior and posterior margin bristles and this is not associated with apoptotic cell death (Abbott, 1986). However, there is a reduction in the number of cells in the wing margin that manifests itself by

erosion of the wing margin (Figure 3.5 B). *Ly* mutations have been shown to be dominant gain of function alleles of *sens*, in that in a *Ly* background *sens* is ectopically expressed (Nolo et al., 2000). To see if *Ly* and *sd* interact genetically, wings were examined from sd^{ETX4} males that were also heterozygous for *Ly*. Flies harboring mutants of both genes show a significant enhancement of the wing phenotype compared to flies with either mutant alone (Figure 3.5 A,B versus C). In the transheterozygous fly the margin bristles are completely absent (Figure 3.5 C), suggesting that these two genes work through a common pathway.

Because *Ly* mutations are gain of function alleles of *sens* and because *Ly* interacts with *sd* it is possible that this could result in alterations of SENS protein levels in *sd* mutant wing discs. Wing discs derived from wild-type flies (Figure 3.6 A) and from flies harboring sd^{58} (Figure 3.6 B,C,D) were stained with an anti-SENS antibody. In wild type discs, SENS is localized to the region fated to become the wing margin with higher levels at the anterior margin in SOP cells, in agreement with previous results (Nolo et al., 2000; and Figure 3.6 A). In addition, *sens* is also expressed in other SOPs distributed throughout the wing disc. In sd^{58} discs, the wing margin-specific expression of *sens* is completely lost (Figure 3.6 C,D), but expression in other SOPs is unaffected. Substantial margin-specific expression is restored when the *vg::sd*-TEA fusion construct is expressed in sd^{58} discs using a *vg*-Gal4 driver (Figure 3.6 E,F,G). That this restoration of SENS is not complete could be attributed to the amount of the fusion *vg::sd*-TEA protein being produced from the transgene. However, this level of restoration is consistent with the notion that the fusion construct can restore the margin specific expression of *wg*, and emphasizes the involvement of *wg* in specifying the formation of SOPs. The mutual

enhancement of mutant wing phenotypes by *sd* and *Ly* mutations can also be explained based on the role of *wg* in SOP formation. As previously mentioned, *Ly* mutations are gain of function alleles of *sens* and are associated with a repression of *wg* expression in domains of high *sens* expression (Nolo et al., 2001). Because *sd* mutations affect the margin-specific expression of *wg*, and in *Ly* mutations there is a repression of *wg* expression, it is predictable that in transheterozygotes the overall WG signal is further reduced at the margin, resulting in the phenotypic enhancement of wing margin loss.

sens needs _sd_ function for sensory organ precursor development.

sens has been shown to be both necessary and sufficient for the formation of organs of the peripheral nervous system (PNS) (Nolo et al., 2000). Ectopic expression of *sens* can result in the formation of extra sensory bristles on the wing and thorax. This ectopic formation of sensory bristles can also happen in the absence of genes of the *achaete-scute* complex, though to a lesser extent (Nolo et al., 2000). To see if *sd* has any role in formation of sensory bristles by ectopic expression of *sens*, and to confirm that *sens* is necessary and sufficient for formation of the sensory bristles as previously reported, *sens* was expressed in a *sd* mutant background. The UAS-*sens* transgene was expressed in both sd^{ETX4} and sd^{58} mutant backgrounds using a *vg*-Gal4 driver and expression from the UAS-*sens* transgene was determined by staining wing discs with the anti-SENS antibody as a control (not shown). If *sd* has no role in ectopic bristle formation by *sens*, then expression of *sens* should result in formation of the sensory bristles missing in the margin of the *sd* mutants. However, *sens* expression is unable to restore the margin specific bristles in *sd* mutants, suggesting that *sens* may need *sd* function for formation of bristles and for proper SOP differentiation. Instead of the

formation of ectopic bristles, expression of *sens* in sd^{ETX4} enhances the wing phenotype (Figure 3.5 D) to resemble the enhancement of sd^{ETX4} caused by a *Ly* mutant (Figure 3.5 C). To test this further, UAS-*sens* was also expressed under the control of a *dpp*-Gal4 construct that drives expression at the Anterior (A)/ Posterior (P) compartment border away from the margin (Figure 3.1 D). Wild type wings expressing *sens* at the A/P border fail to inflate properly upon eclosion (Figure 3.5 E,F) but exhibit numerous ectopic bristles at the position of the A/P border (arrowheads in 3.5 E,F) as well as numerous ectopic bristles on the thorax. Expression of *sens* in a sd^{58} mutant background, however, results in very little to no ectopic bristle formation at the A/P border (Figure 3.5 G,H), again suggesting that *sens* possibly needs *sd* function for formation of SOPs.

In conclusion, a further characterization of the functions of the SD/VG complex during wing development is reported by analyzing the roles of *sd*, via the *vg*::*sd*-TEA fusion during patterning by *wg*, during growth, and during SOP development. In the narrow context of the D/V specific expression of *wg*, the SD/VG complex appears to act upstream of *wg* as evidenced by the rescue of the D/V WG stripe by the fusion construct and the rescue of *sd* wing mutations by the expression of exogenous WG. In addition, the relationship between *sd* and *sens* in the development of margin-specific bristles is clarified and the results show that *sens* needs *sd* function for proper development of the PNS organs. Our current model (Figure 3.7) for actions of the SD/VG complex during wing development, incorporating the new data herein, is that the SD/VG complex either induces or maintains the expression of WG. This, in turn, causes expression of SD and VG to promote cell proliferation in the wing pouch. At the D/V boundary WG also

mediates the expression of *sens* via its actions on the *achaete scute (AS-C)* complex that, in the presence of SD, helps to specify the SOP fate.

Materials and Methods

Stocks and Crosses:

All crosses were carried out at 25°C. *vg*-Gal4 drives expression of a UAS transgene in a *vg*-Boundary enhancer pattern and was initially created by Morimura and Hoffman (unpublished results) and obtained from Sean Carroll. UAS-*vg*::*sd*-TEA mimics the native SD/VG transcription complex and is described in Srivastava et al., (2002) . UAS-*wg* was obtained from Andrew Simmonds (Simmonds et al, 2001). UAS-$dTCF^{DN}$ is described in Cadigan et al., (1998) and was a gift from Ken Cadigan. The *C96*-Gal4 driver was obtained from Gabrielle Boulianne and directs expression of a transgene at the D/V border in the wing disc. *dpp*-Gal4 was obtained from Ken Irvine and drives expression along the A/P border in the wing. Ly^1 is described in Flybase (**http://flybase.bio.indiana.edu/**).

Antibody staining:

The VG antibody is described and antibody staining was performed according to a published protocol (Williams et al, 1991), and was visualized using a Leica TCS confocal microscope. The anti-SENS antibody was used as described in (Nolo et al., 2000). Mouse anti-WG antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Anti-PH3 antibody was from Upstate Biotech and has been previously described (Hendzel et al., 1997). Secondary antibodies were anti-rabbit Alexa 488 from Molecular Probes and Cy3 conjugated anti-rabbit, anti-guinea pig and anti-mouse IgG from Jackson Immunoresearch.

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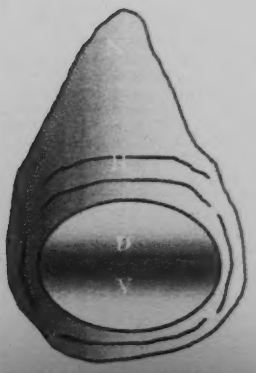
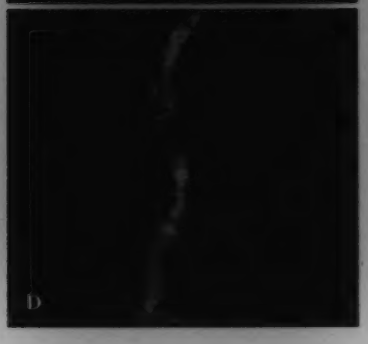
Figure 3.1. Schematic of different regions of the wing disc and expression patterns of the Gal4 drivers used in this study visualized by monitoring expression of a UAS-GFP transgene. A) Schematic of the wing disc showing the different regions. Dorsal and Ventral areas in the wing pouch are marked as D and V, respectively, on either side of the D/V border. N refers to the notum region and H refers to the hinge region. B-D) Wing discs expressing UAS-GFP under the control of different Gal4 drivers. B) *C-96*-Gal4 driving expression strictly along the D/V border (pseudo-colored Blue, the nuclei are stained by DAPI to visualize the wing disc outline better and are pseudo-colored red). C) *vg*-Gal4 driving expression along the D/V border as well as hinge regions of the wing disc. D) *dpp*-Gal4 driving expression along the A/P border.



A

B

C



D

Figure 3.2. Rescue of the D/V WG stripe by expression of the *vg::sd*-TEA domain fusion. A-I) Wing discs from late third instar larvae, with dorsal facing up and ventral facing down, were stained for VG (Green A,D,G) and WG (Red B,E,H). The overlap is shown in panels C,F and I. A,B,C). Wing discs derived from wild type larvae demonstrate the spatial relationship between VG and WG. Note the localization of WG (red channel) along the D/V border and around the wing pouch in cells fated to give rise to hinge. In addition, expression in the notum is also seen. The expression of VG is seen throughout the wing pouch, with more protein along the D/V stripe of WG localization. Panel C shows clearly the overlap of VG with WG along the D/V boundary. D,E,F) Loss of D/V WG stripe in *sd* mutations. In wing discs derived from male sd^{58} mutant flies (D) the reduction in VG (green channel) is evident. The loss of the D/V stripe of WG is evident in E. This can be best visualized by comparing C and F. In F, there is no overlap between WG and VG along the D/V boundary. G,H,I) Restoration of the D/V WG stripe by expression of the fusion construct (UAS*vg::sd*-TEA) under control of a *vg*-Gal4 driver. Compare E to H and F to I.

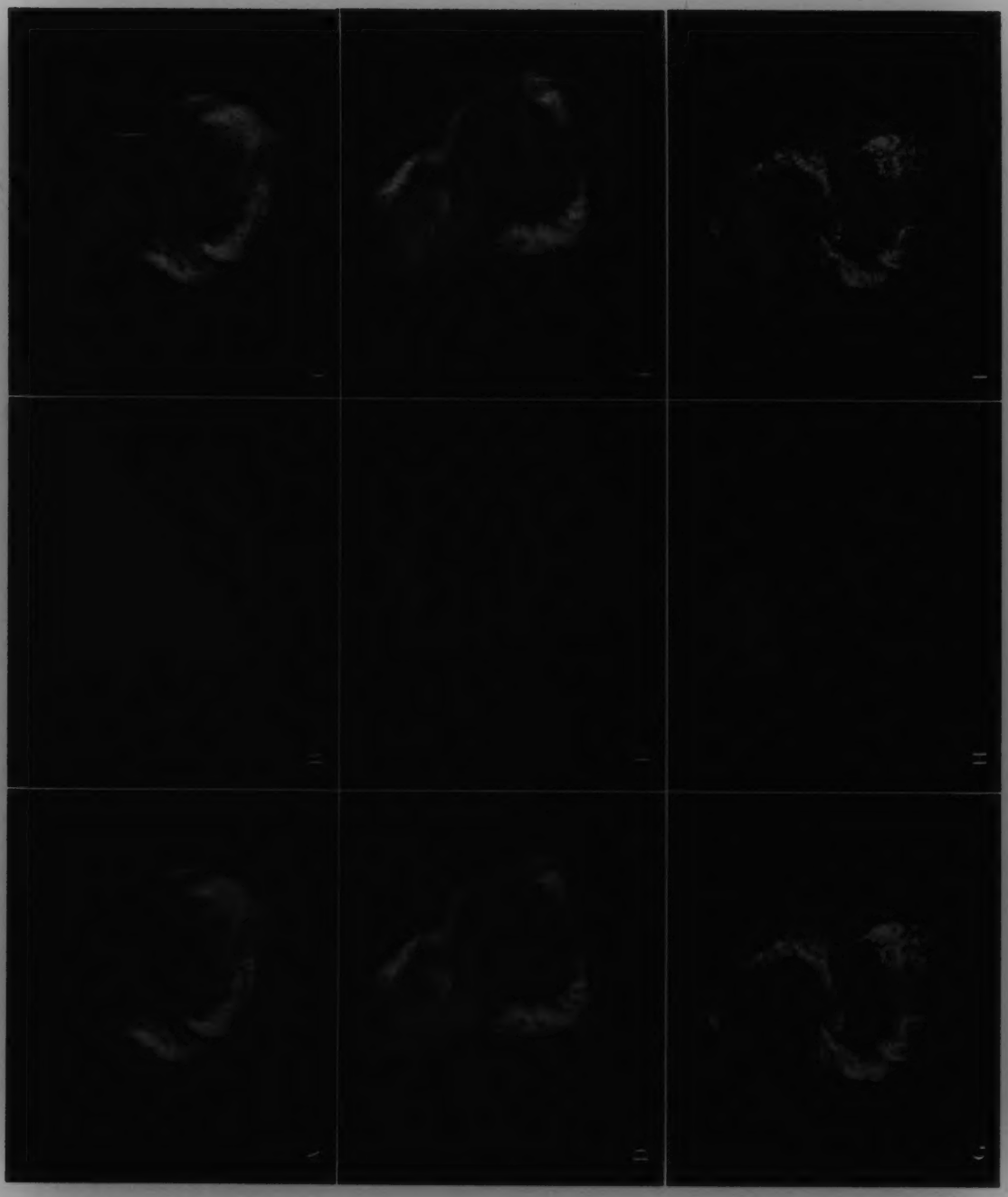


Figure 3.3. The SD/VG complex promotes cellular proliferation.

A-C) Late third instar wing discs stained with an anti-PH3 antibody to visualize mitotically active cells in different genetic backgrounds. A) Wild type wing disc showing numerous mitotically active cells in the wing pouch. B) a sd^{58} wing disc showing a reduced number of mitotically active cells in the area of the wing pouch. C) a sd^{58} wing disc expressing the fusion construct. Note the restoration of mitotic figures to wild type levels in C.

Figure 3.4. Rescue of a *sd* mutation by exogenous WG.

A-F) Whole mounts of adult wings from the following genotypes: A) Oregon-R wild-type. B) Wing from a fly expressing a dominant negative form of dTCF at the D/V border under the control of a C96Gal4 driver. Notice the reduction in the size of the wings. C) Male sd^{ETX4} wing showing the characteristic notching associated with a *sd* mutation. This wing is relatively similar to that in B in the sense that both have notches and missing margin elements. D) Wing from a male fly over-expressing *wg* in a sd^{ETX4} background under the control of a *vg*-Gal4 driver. This shows rescue compared to the wing in C. E) Wing from a male sd^{58} fly. Notice the reduction in size of the wings compared to A or C. F) Over-expression of *wg* using a *vg*-Gal4 driver in a sd^{58} background results in a partial rescue of the wing phenotype. Compare wings in E and F. All wings in this figure are shown at the same magnification.

A
B
C
D
E
F

Figure 3.5. Genetic interaction between *sens* and *sd*.

A-D) Whole mounts of wings from adult flies. E-H) Scanning electron micrographs (SEMs) of adult wings. A) Wing from a sd^{ETX4} male fly. Note the notching in the wings accompanied with the loss of margin bristles. B) Wing from a *Ly*/+ fly. Note the loss of wing margin. C) Wing from a *Ly*/+; sd^{ETX4} male fly. Notice the enhancement of the wing phenotype as compared to wings in A and B. D) Wing from a male fly of genotype UAS-*sens*C5/*vg*-Gal4; sd^{ETX4} /Y. Notice the enhancement of the sd^{ETX4} phenotype and also the loss of all the bristles from the margin. E) SEM of an adult wing expressing *sens* under the control of a *dpp*-Gal4 driver. Adult wings from this genotype fail to inflate properly after eclosion, but notice the numerous ectopic bristles produced as a result of exogenous *sens* expression (arrowheads). Arrows point to the normal wing margin. F) is an enlargement of a region from the wing in E to show bristle details. G) SEM of an adult wing expressing *sens* in a sd^{58} mutant background and under the control of a *dpp*-Gal4 driver. H) is an enlargement of the wing tip in G. Notice that there are very few ectopic bristles in this genetic background.

B
D
A
C

Figure 3.6. Expression of the *vg::sd*-TEA fusion construct restores SENS expression in wing discs derived from *sd* mutant animals.

A-G) Wing discs derived from late third instar larvae stained for VG (green channel) and SENS (red channel). Anterior is to the left and dorsal is facing the top of the page.

A) Localization of SENS in wild-type discs. In addition to being expressed in sensory organ precursors in the disc, *sens* is also expressed in the SOPs along the D/V boundary, with higher expression in the anterior half. B-D) Wing discs from sd^{58} animals showing the reduction of VG in B and loss of the D/V *sens* expression stripe in C. D) Overlap between B and C highlights the loss of D/V *sens* expression. E-G) Wing discs from sd^{58} animals expressing the fusion construct under control of a *vg*-Gal4 driver. Notice the restoration of *sens* expression in F and this is better visualized by comparing the overlap panels D and G. Panel E shows that normal *vg* expression is restored with the fusion construct.

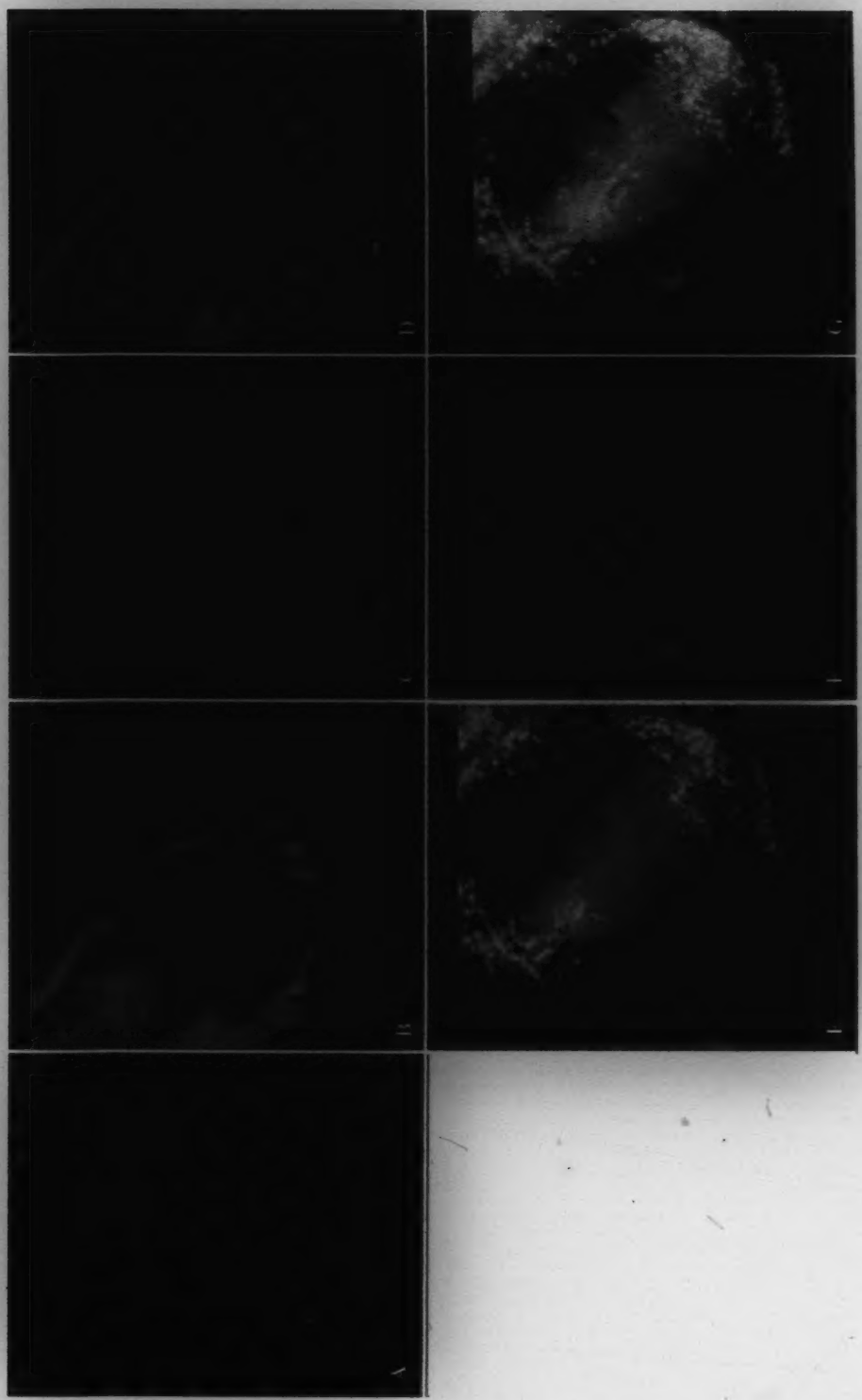
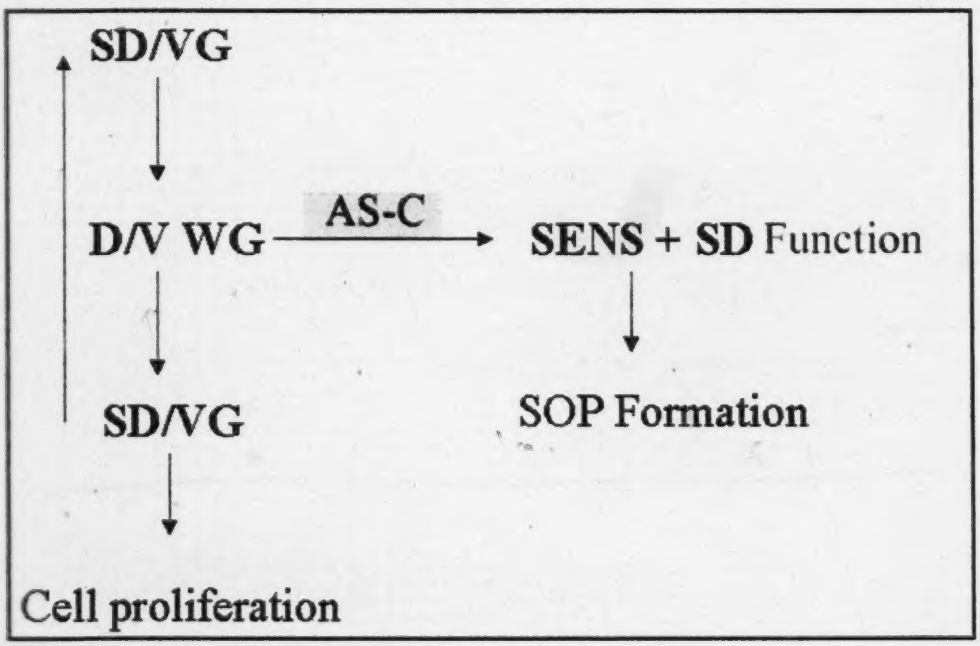


Figure 3.7. A model depicting the relationships between WG, SD/VG and SENS during wing development. The SD/VG complex is responsible for either the maintenance or induction of the D/V WG expression. The D/V WG in turn regulates SD and VG which autoregulate each other and are responsible for proliferation of cells within the wing pouch. The D/V WG also activates SENS through AS-C, and it appears that SENS needs SD function for specifying SOP fate.


SD/VG
D/V WG
AS-C
SENS + SD Function
SD/VG
SOP Formation
Cell proliferation

Chapter 4

Functional dissection of the TEA domain region found in a VG/SD-TEA chimera during *Drosophila* wing development.

Introduction

Numerous genetic and molecular studies have shown the importance of *scalloped* (*sd*) and *vestigial* (*vg*) during wing development in Drosophila (Bray, 1999; Campbell et al., 1992; Campbell et al., 1991; Guss et al., 2001; Halder and Carroll, 2001; Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002; Varadarajan and VijayRaghavan, 1999). The *vestigial* protein (VG) contains multiple domains which aid in its function and, together with the *scalloped* protein (SD), forms a functional transcription complex important for wing development (Guss et al., 2001; Halder et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002). SD binds to DNA via an evolutionarily conserved TEA DNA binding domain that is made up of three helices (Burglin, 1991). The vertebrate homologue of *sd*, TEF-1, can substitute for *sd* function during wing development (Deshpande et al., 1997) and has been shown to have a function during cardiac (Farrance et al., 1992; Gupta et al., 1997) and skeletal muscle development (Carson et al., 1996; Jacquemin et al., 1996; Maeda et al., 2002) as well as neural development (Yasunami et al., 1995). Thus, studying the properties of *sd* in Drosophila offers the possibility of extrapolating any insights gained to TEF-1. Mutations of *sd* may be viable or lethal, thereby suggesting that the SD protein is important for a function that is necessary for viability of the fly (Campbell et al., 1992; Campbell et al., 1991). It has recently been shown that a mutation within the TEA DNA binding domain of SD results in pupal lethality as well as a defect in wing development, highlighting the

in vivo importance of this domain (Chapter 5). Furthermore, a domain in the C-terminal half of SD has also been demonstrated to be important for binding VG (Simmonds et al., 1998; Vaudin et al., 1999) and for viability (Chapter 5).

A region in the SD protein extending from amino acid residues 63-211 (Chapter 2) has recently been identified to be necessary and sufficient for development of the wing in Drosophila (Srivastava et al., 2002). It was also shown that this region, containing the TEA DNA binding domain, also contains a Nuclear Localization Signal (NLS) that appears to be indirectly responsible for also carrying VG into the nucleus. This was demonstrated by using a construct that consisted of a fusion between the full-length *vg* gene and the *sd* TEA domain. This fusion construct is able to mimic the native VG/SD transcription complex (Srivastava et al., 2002). The conserved TEA DNA binding domain has been previously reported to have three helices (Figure 4.1) and most of the knowledge about the TEA domain comes from *in vitro* studies on vertebrate TEF-1 (Burglin, 1991; Hwang et al., 1993).

Herein, *in vivo* evidence is presented that further localizes the critical region of SD needed for wing development from amino acids 63-211 of SD present in the original fusion construct to amino acids 87-185. It is also shown that this region retains a functional NLS, as assayed by the localization of VG to the nucleus. A dissection of the TEA domain contained within this region should further our understanding of the role of the various helices in the TEA DNA binding domain. It would also solidify the notion that the NLS in SD resides within the TEA domain.

Results and Discussion

Construction of deletions used in this study.

To more precisely define the region in SD needed for nuclear localization, as assayed via the localization of VG, and to identify the critical region of SD needed for proper functioning of the VG/SD transcription complex *in vivo*, a series of constructs was created to subdivide the TEA domain. A combination of 3' deletions of the UAS-*vg::sd*-TEA fusion construct as well as a 5' deletion of SD into the TEA domain was used. All of the rescue experiments were carried out using the UAS/Gal4 system (Brand and Perrimon, 1993). The original fusion construct contained an entire *vg* gene fused in frame to the *sd* TEA domain that encoded amino acids 63-211 of the SD protein (Chapter 2). The 3' deletion constructs (made in the original fusion described above) used are shown in Figure 4.1 A as UAS-*vg::sd*-TEA del1 and UAS-*vg::sd*-TEA del2, and will be referred to herein as del1 and del2. Del1 retains SD amino acids 63-185 whereas del2 retains SD amino acids 63-152 and eliminates half of the putative NLS as well as perturbing helix3 of the TEA DNA binding domain (Figure 4.1 A,B). Thus, these two constructs will help to define the 3' limits of the TEA DNA binding domain of SD needed for this function, as well as further characterize the NLS. A 5' deletion of SD is also used that eliminates the N-terminal 87 amino acids to the first helix of the SD TEA domain. This will be useful in further defining the N-terminal limit of SD needed for its DNA-binding function (Figure 4.1 A). Full-length *vg* and *sd* constructs are also used in this study as controls.

The functionality of the 3' deletions in the fusion construct was tested based on the following criterion. Over-expression of *vg* in a genetic background also harbouring a *sd* enhancer trap has been shown to cause ectopic expression of *sd* as assayed by β-

galactosidase staining (Simmonds et al., 1998) possibly by binding of VG/SD to enhancer elements within the *sd* locus. Retention of this function by the 3' deletions of the fusion construct was judged by over-expressing the deletion constructs in a sd^{ETX4} background along the anterior posterior border of the wing disc using a *ptc*-Gal4 driver. If, indeed, the deletion constructs retain the relevant *sd* function they should be able to cause ectopic expression from the *sd* enhancer trap via the still intact *vg* in the 3' deleted fusion constructs. UAS-*vg*::*sd*-TEA *del1* does give ectopic expression of *sd* (data not shown). Hence, the truncated fusion protein is being transcribed and translated so that the *vg* gene in the fusion construct must be functioning. The UAS-*vg*::*sd*-TEA *del1* construct is missing encoded SD amino acid residues 186-211 that were present in the original fusion construct. UAS-*vg*::*sd*-TEA *del2*, on the other hand, fails to give an ectopic stripe (data not shown) suggesting that this construct is incapable of allowing the full length *vg* in the fusion product to function. It is also possible that the point of insertion of this construct in the genome results in silencing of the transgene. The possibility of such a position effect was ruled out by examining ten independent insertions. All of them fail to give an ectopic expression of *sd,* suggesting that the product from this construct is non-functional. Thus there is no functional VG produced by this construct and hence no ectopic induction of the *sd* enhancer trap. The UAS-vg::*sd*-TEA *del2* construct partially eliminates the third helix of the TEA DNA binding domain and could result in a product that is unstable and rapidly degraded.

The constructs described above were used in two assays to define more precisely the critical region within the SD protein needed for its function in wing development. Firstly, they were tested for their ability to carry VG into the nucleus, as assayed by

nuclear localization of VG, and secondly their functionality was tested by assaying for the ability to rescue a *sd* wing mutant phenotype.

Region in SD needed for nuclear localization of VG.

It is known that VG is a nuclear protein (Williams et al., 1991) (Figure2A-A") that needs SD for its nuclear localization (Halder et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002). It has also been reported that over expression of wild-type *vg* in wing discs in a *sd* mutant background results in the cytoplasmic localization of this exogenous VG (Srivastava et al., 2002) and this result is repeated in Figure 4.2 B-B". However, co-expression of wild type *vg* and *sd* in a *sd* mutant background results in nuclear accumulation of VG (data not shown). Furthermore, expression of a fusion between *vg* and the *sd* TEA domain (amino acids 63-211 of SD) in *sd* mutant wing discs also results in the nuclear localization of VG (Srivastava et al., 2002). Based on this, it was proposed that the *sd* TEA DNA binding domain also encoded a segment, within this 63-211 amino acid motif, responsible for the nuclear localization of SD and hence VG, by virtue of its ability to bind SD. Of course the protein from the fusion construct has a full length VG as part of it and, thus, doesn't need to rely on this binding to form a SD/VG complex. Co-expression of *vg* (from a transgene) and a construct carrying an N-terminal deletion of 87 amino acids of SD in a *sd* mutant wing disc still facilitates the nuclear accumulation of VG (Figure 4.2 C-C" compared to 4.2 B-B"). This implies that the first 87 amino acids of the SD protein are dispensable with respect to the nuclear localization of SD, and hence VG also. Since this truncation keeps the VG binding domain of SD intact, the over-expressed VG is able to bind to the truncated SD and get into the nucleus.

To further localize the NLS motif within the TEA domain of SD, 3' deletions of the original fusion construct were also used. UAS-*vg*::*sd*-TEA *del1* was expressed in a sd^{58} mutant background using a *vg*-Gal4 driver. Wing discs from sd^{58} mutant animals expressing the above construct were stained with an anti VG antibody. The data demonstrate that this 3' deletion (from the 63-211 portion of SD included in the original fusion) also retains the ability to promote the nuclear localization of VG (Figure 4.2 D-D"). This del 1 construct retains a portion of *sd* encoding amino acids 63-185. Together with the 5' deletion results, one can conclude that the *sd* TEA domain region found in the fusion construct (63-211 amino acids from SD in the fusion protein) needed for nuclear localization resides within amino acids 87-185 of SD. This coincides with the original identification of a NLS in this region of SD (Srivastava et al., 2002).

N-terminal region in SD needed for wing development.

The region of the SD protein from amino acids 63-211 has been previously shown to be critical for wing development, in that a fusion between VG and this region of SD is necessary and sufficient for wing development. The fusion protein mimics the native VG/SD complex and is able to rescue *sd* wing mutations as well as *sd*, *vg* double mutants. To define the minimal region needed for wing development more precisely, the constructs shown in Figure 4.1 A were assayed for their ability to rescue a sd^{58} wing mutation.

As expected, the co-expression of full-length *vg* and full length *sd* using a *vg*-Gal4 driver is able to rescue the wing phenotype associated with the hypomorphic phenotype of a sd^{58d} (Figure 4.3-A-C) hemizygous fly. However, the rescue by this co-expression (Figure 4.3 C) is not as complete as when only full-length wild-type *sd* is expressed (data

not shown). It is important to point out that full length *sd* on it's own is capable of completely rescuing the sd^{58} mutation to wild type levels (Figure 4.3 A). This result highlights the importance of a proper balance between the levels of SD and VG, so that an alteration of the normal levels results in an incomplete rescue. Co-expression of *vg* with a *sd* deletion that encodes an N-terminal 87 amino acid truncation results in a level of rescue of sd^{58} (Figure 4.3 D) that is similar to the rescue obtained by expressing wild type *sd* and *vg* (compare Figure 4.3 C-D). This suggests that the N-terminal 87 amino acids of SD are not critical for wing development and this truncated version of SD is capable of forming a transcription complex that is similar in function to a wild type complex. Further support for this comes from another observation where co-expression of *vg* and *sd* in a wild type background (data not shown) or a sd^{58d} background results in missing sensory bristles from the margin (Figure 4.3 C). This phenotype is also seen when *sd* is expressed on its own in a wild type background but not when *vg* is expressed alone (data not shown). This may be interpreted to mean that the level of SD is limiting for wing development and the level of VG is not. Thus, the expression of *sd* results in more SD/VG complex than is needed, resulting in the dominant negative phenotype of bristle defect at the margin. This margin bristle phenotype is also observed at the wing margin when the 87 amino acid truncation of *sd* is expressed in a wild type background using a *vg*-Gal4 driver (Figure 4.3 G and I). This further suggests that the N-terminal 87 amino acid truncation of SD can still form a transcription complex as functional as the wild type SD/VG complex. It was previously shown that a fusion between *vg* and the *sd* TEA (amino acids 63-211) domain is capable of rescuing a *sd* mutation (Srivastava et al., 2002). When this fusion construct is expressed in a wild type background the loss of

margin bristles is similar to the one observed when *sd* is expressed on it's own (Figure 4.3 F and H) or when *sd* is co-expressed with *vg* (Figure 4.3 C). This suggests that this fusion construct is as functional as the native complex. Expression of a C-terminal truncation of this fusion construct (but still encoding SD amino acids 63-185) in a sd^{58} mutant background results in substantial rescue of the wing phenotype at room temperature, but not as complete as reported for the original fusion construct (Figure 4.3 E). There are at least two possibilities to explain why the construct encoding only the 63-185 amino acid portion of SD is less functional than the fusion construct that encodes the 63-211 amino acid portion of the SD protein. Firstly, it is possible that this del1 construct when expressed does not bind DNA as well as the original fusion construct encoding amino acids 63-211 of SD and thus is less efficient in activating transcription. Secondly, it is possible that the amount of protein produced from the del1 construct is lower compared to the original fusion construct so that the level of transcriptional activation by this construct is not the same as using the original fusion construct.

The first possibility is favoured because the del1 construct is unable to give the dominant-negative bristle loss phenotype that is associated with the intact fusion construct (Figure 4.3 F and arrows in H) both at room temperature as well as at 29°C. However, the rescue of the *sd* mutation is comparable to the one by the original fusion construct when the experiment is conducted at 29°C. It is known that higher temperatures result in more Gal4 expression and hence more transcription of genes downstream of a UAS element (Speicher et al., 1994).This suggests that the del1 construct is able to produce an amount of protein which is comparable to the levels produced by the original fusion construct, so that in the absence of wild type SD/VG complex (in sd^{58d}) this

truncated fusion is able to substitute for the wild type SD/VG complex function. It is possible that in wild type wings a wild type SD/VG complex is already present and has a stronger affinity for binding DNA compared to the del1 truncated fusion. Thus, the truncated fusion is unable to bind to the DNA elements in the presence of wild type SD/VG complex, and no dominant-negative bristle phenotype is produced. Because it was determined that the del2 construct was non-functional as assayed by its inability to induce ectopic expression from a *sd* enhancer trap, data from this construct are not presented.

Based on the results presented herein, the region of the SD protein that is necessary for nuclear localization and also for the functionality of the SD/VG transcription complex can now be said to reside within the portion of the SD protein from amino acids 87-185.

Materials and Methods

Stocks and Crosses.

All crosses were carried out at room temperature (~24°C) except where specified. The *vg*-Gal4 construct has been previously described and drives expression in the wing pouch along the dorsal/ventral compartment border. UAS-*sd* and UAS-*vg* are described in Simmonds et al. (1998) and were a gift from Ken Irvine. UAS-*vg*::*sd*-TEA is described in Srivastava et al. (2002).

Construction of the deletion constructs and transgenic flies.

The 3' deletions in the UAS-*vg*::*sd*-TEA fusion were made by PCR amplification of the region represented in each construct (Figure 4.1 A) using upstream and downstream primers that had engineered *Bam*H1 sites in them. The UAS-*vg*::*sd*-TEA

fusion construct was used as the template for PCR reactions. The PCR-amplified product was digested with *Bam*H1 and then cloned into *Bgl*11-digested and shrimp alkaline phosphatase-treated pUAST. The constructs were then sequenced for verification and injected into *yw* flies with a helper plasmid as the source of transposase. The construct bearing the N terminal 87 amino acid truncation of *sd* was obtained from Leola Chow and will be described elsewhere.

Antibody staining

All antibody staining was carried out using a protocol described previously (Williams et al., 1991). A rabbit anti-VG antibody was used for localization of the VG protein and nuclei were stained using DAPI. The secondary antibody was a Cy3-conjugated anti rabbit antibody from Jackson Immunoresearch. All preparations were viewed using a Leica TCS SP2 Confocal microscope.

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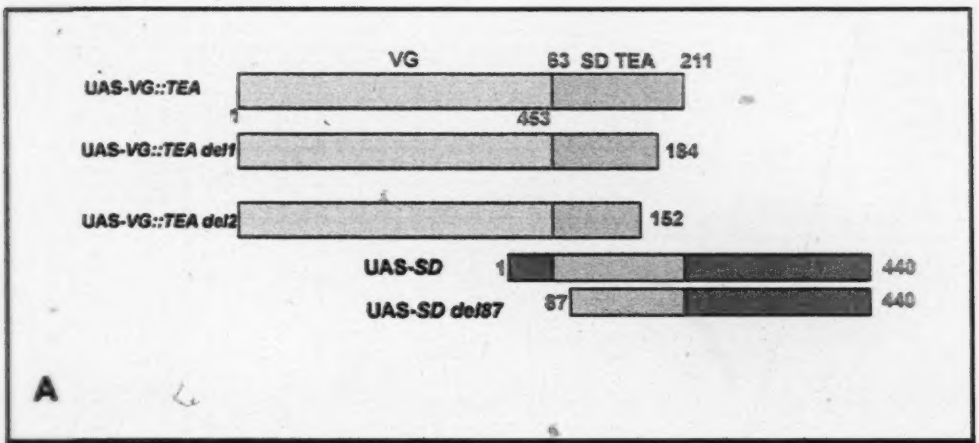
Figure 4.1. Various constructs used in this study and features of the SD TEA domain. A) Schematic of the various UAS constructs used in this study. The light blue rectangle represents a complete VG (amino acids 1-453) protein and the dark green rectangle represents the TEA domain of SD found in the various constructs. The extent of each region is shown by the corresponding amino acid numbers. UAS-*vg::sd*-TEA refers to the fusion of *vg* (encoding amino acids 1-453) to *sd* (encoding amino acids 63-211 that contains the TEA domain) and described in Srivastava et al. (2002). UAS-*vg::sd*-TEA del1, is a C-terminal truncation of the fusion construct and encodes SD from amino acids 63-185. UAS-*vg::sd* TEA del2 is a larger deletion and encodes SD from amino acids 63-152. The full-length *sd* construct is represented by a red rectangle, plus the corresponding region found in the fusion construct represented by a dark green rectangle. UAS-*sd* del87 carries an N-terminal truncation of a segment encoding the first 87 amino acids of the SD protein but retaining the remaining portion of SD from residues 88-440. B) Amino acid sequence of the SD TEA domain containing region showing various features. Green, Magenta and Orange rectangles refer to helix1, 2, 3, respectively, of the TEA domain as described (Burglin, 1991). The amino acid sequence of the putative NLS is shown in red and various putative protein phosphorylation sites are indicated by bars under the corresponding amino acids.



63 NGSMVDSKNLDVGDMSDDEKDLSSA

DAEGVWSPDIEQSFQEALSIYPPCGR

RKIILSDEGKMYGRNELIARYIKLRTGK

TRTRKQVSSHIQVLARRKLREIQAKIK

VQFWQPGLQPSTSQDFYDYSIKPFPQ

PPYPAGKTSTAVSGDET 211

B

Figure 4.2. Nuclear localization of VG in discs expressing various constructs.

The assay for a functional SD NLS is the ability of VG to enter the nucleus since it is well established that VG enters the nucleus by virtue of its ability to bind SD. Thus a non-functional SD NLS would also have VG localized to the cytoplasm.

A-D") Confocal scans of wing discs stained for VG (red) and nuclei (Blue). In all panels ' refers to the blue channel and " refers to the merge of the two channels. A-A") VG localization in wild type wing discs showing nuclear localization as judged by the punctate nature of localization and also by the magenta colour produced from the overlap of the blue and the red channels. B-B") Consequences of expressing *vg* in a sd^{58} background using a *vg*-Gal4 driver. VG is cytoplasmic in these discs. C-C") Wing discs co-expressing VG and an N-terminal 87 amino acid truncation of SD in a sd^{58} mutant background. VG in these discs is also punctate and nuclear in nature. D-D") Wing discs expressing a C-terminal truncation (del1) of the fusion construct in a sd^{58} mutant background. VG in these discs is also nuclear in nature. Because del2 was determined to be non-functional (with respect to its ability to ectopically express *sd*) the nuclear localization data for it are not presented.

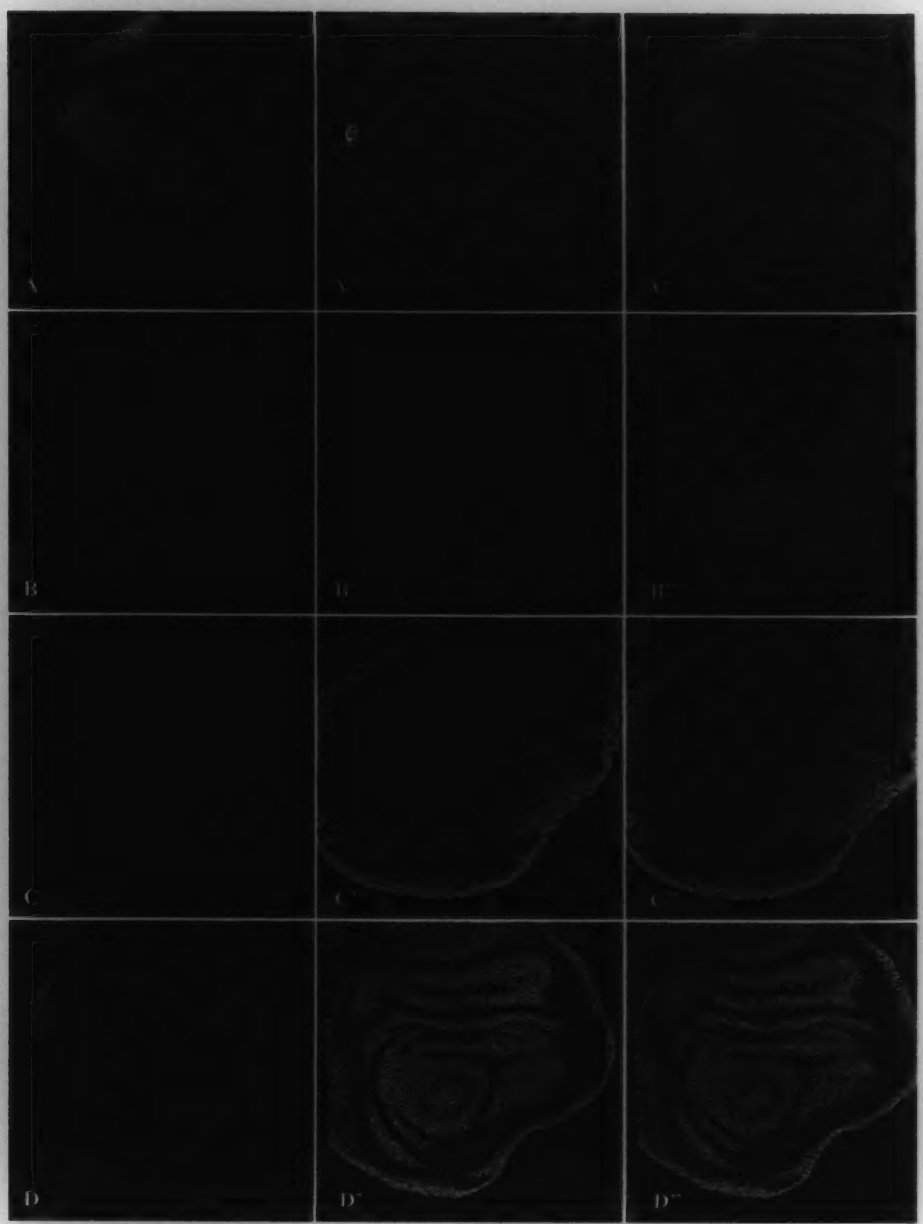
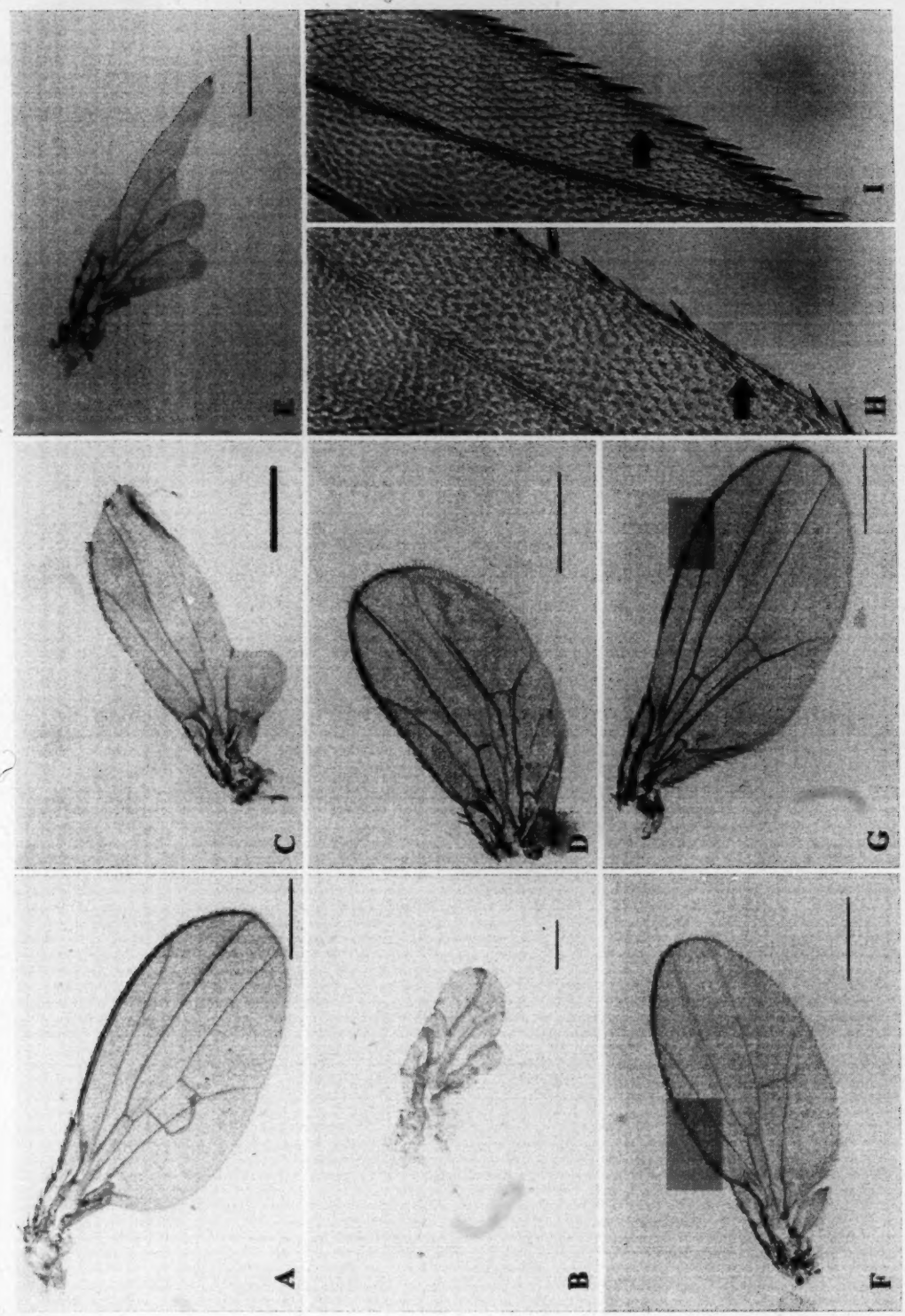


Figure 4.3. Wing phenotypes associated with expression of full length *sd* and *sd* deletion constructs.

A-I) Whole mounts of adult wings expressing full length, N-terminal 87 amino acid truncated version of *sd* with *vg*, or different versions of the *vg::sd*-TEA fusion construct. A) Wild type wing B) Wing from a male sd^{58} fly. Notice the difference in the sizes of the wings in A and B. C) Partially rescued wing from a male sd^{58} fly co-expressing *sd* and *vg* under the control of a *vg*-Gal4 driver. D) Rescued wing from a male sd^{58} fly co-expressing *vg* and an N-terminal 87 amino acid truncated form of SD. Notice the substantial level of rescue in C and D. E) Wings from sd^{58} males showing the substantial amount of rescue obtained by expressing UAS-*vg::sd*-TEA del1 using a *vg*-Gal4 driver at 25°C. This rescue is much better when the rescue experiment is carried out at 29°C (data not shown). Rescue by del2 is not shown as this construct is non-functional. F) Wing from a female fly expressing the original fusion construct in an otherwise wild type background. Notice the erosion of the margin and loss of bristles as compared to wing in A. The rectangular shaded box highlights this feature in H (arrow). G) Wing from a female fly expressing the N-terminal 87 amino acids truncated form of SD. Notice the loss of bristles at the margin also shown in I (arrow).


A
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I

Chapter 5

Identification of a VESTIGIAL binding domain by molecular analysis of *scalloped* recessive lethal alleles in *Drosophila.*

Introduction

The *scalloped (sd)* gene was first identified because of mutations which resulted in a loss of wing margin structures and gave the resulting wing a scalloped appearance (Daniels et al., 1985; Grunberg, 1929). Since its first identification, *sd* alleles have also been identified in screens for mutations affecting wings and chemoreception (Inamdar et al., 1993). With respect to the wing phenotype, both strong and weak *sd* alleles exist that are viable as homozygotes. While strong alleles cause the wings to be reduced in size, as in sd^{58d}, weak alleles result only in partial loss of the wing margin structures, as in sd^{ETX4} (Campbell et al., 1991). The viable mutations of *sd* exhibit a wing phenotype and those that have been characterized appear to affect regulatory elements (Campbell et al., 1991). Thus, these provide little information about the molecular nature of the protein.

The *sd* locus encodes multiple transcripts thought to be products of alternative splicing events (Campbell et al., 1991). The E21 isoform (Campbell et al., 1992; Campbell et al., 1991) is the best studied so far and encodes a protein product of 440 amino acids. This protein belongs to a family of highly conserved transcription factors containing the TEA/ATTS DNA binding domain (Burglin, 1991). *In silico* studies and *in vitro* binding experiments have subdivided the E21 isoform crudely into two domains (Simmonds et al., 1998). The N-terminal region contains the TEA/ATTS DNA binding domain (Campbell et al., 1992). The evolutionarily conserved C-terminal region has of yet not

been demonstrated to contain a recognizable domain except one which is necessary for binding VESTIGIAL (VG) *in vitro* (Simmonds et al., 1998).

The *sd* gene is expressed in the embryonic nervous system and in the larval wing, eye-antennal and leg discs implicating it in some important role during development of these structures (Campbell et al., 1992; Campbell et al., 1991). Furthermore, the existence of lethal alleles (Campbell et al., 1991) lends credence to the notion that *sd* has a vital function in tissues other than the wing. In the wing disc the *sd* gene is expressed in a pattern that encompasses the entire wing pouch (Campbell et al., 1992), overlapping with the expression pattern of the *vestigial* (*vg*) gene product (Williams et al., 1991). The SD and VG proteins bind to each other (Paumard-Rigal et al., 1998; Simmonds et al., 1998) to form a functional transcription complex. In this complex, SD provides the DNA binding activity as well as the nuclear localization signal for VG (Halder et al., 1998; Srivastava et al., 2002) whereas VG provides the activation function. The binding of SD and VG involves a 56 amino acids SD interaction domain (SID) in VG with a C-terminal region of SD (Simmonds et al., 1998). Apart from the SID, VG also harbors two functional domains that provide the activation function of the SD/VG transcription complex (MacKay et al., 2003). In this respect VG appears to act as a transcription intermediary factor (TIF) (Hwang et al., 1993). TIFs are proposed to be tissue-specific molecules that are required to activate transcription by SD in *Drosophila* (Simmonds et al., 1998), and by its homologues in other organisms in tissues in which they are expressed (Hwang et al., 1993). Thus, in addition to VG, many more TIFs may be involved in the activation of transcription by the SD protein in tissues other than the wing. In addition to its function in wing development, VG has also been shown to be

involved in muscle development (Bate and Rushton, 1993; Sudarsan et al., 2001) and it is possible that this function is also mediated by SD.

Homologues of *sd* from several organisms have been cloned and characterized (Aramayo and Timberlake, 1993; Azakie et al., 1996; Chaudhary et al., 1995; Gavrias et al., 1996; Ichi-ishi and Inoue, 1995; Mirabito, 1989; Xiao et al., 1991) and show a remarkable degree of conservation within the DNA binding domain as well as in the C-terminal region. The homologues from other metazoan organisms are expressed in tissues similar to where *sd* is expressed. For example, both *sd* (Campbell et al., 1992) and its vertebrate homologue TEF-1 (Transcription Enhancer Factor-1) are expressed in neural tissues (Yasunami et al., 1995). In addition, TEF-1 has been shown to be important for both cardiac (Gupta et al., 1997; Maeda et al., 2002c; Ueyama et al., 2000) and skeletal muscle development (Fabre-Suver and Hauschka, 1996; Farrance and Ordahl, 1996; Hsu et al., 1996; Stewart et al., 1994; Stewart et al., 1996). The role of a VG human homologue, Vestigial like-2 (Maeda et al., 2002a) in muscle development has also been documented and it has been suggested that TEF-1 may play a role in mammary tumorigenesis by its effects on the mouse mammary tumor virus long terminal repeat (Maeda et al., 2002b). Furthermore, hTEF-1 can substitute for *Drososphila sd* during wing development and is able to rescue *sd* wing mutations (Deshpande et al., 1997). TEF-1 also rescues the lethality associated with a *sd* lethal allele (Deshpande et al., 1997).

Thus, the evidence points to functional conservation of the two genes over the course of evolution. It is logical to believe that studies of *sd* in *Drosophila* will provide valuable insights into the nature and function of homologues in other organisms. Studies

involving the mammalian homologues of *sd* have primarily involved *in vitro* methodologies and the TEA domain has been the focus of intense *in vitro* investigation. While domains in the C-terminal region of TEF-1 have been predicted, their biological function has not been ascertained.

To gain further insights into the function of the *sd* gene product, a molecular characterization of a set of five recessive lethal mutations of the *sd* gene was done. These alleles were previously mapped within the *sd* gene and have been proposed to encode lesions in the C-terminal portion of the SD protein (Campbell et al., 1991). The characterization of the lethal mutations presented herein links specific residues in the SD protein with a phenotypic consequence in the organism. Also, the data show that molecular lesions associated with these alleles help to define a domain in SD responsible for binding VG. Although this domain has been previously predicted (Vaudin et al., 1999), it was based solely on the comparison of the amino acid sequence in TEF1 that binds to TONDU, the human homologue of VG, and comparing the TEF-1 motif to the SD amino acid sequence. It is likely that this VBD overlaps with a domain that is important for satisfying some vital function important for early development of the fly. Finally, it is reported that one of the lethal mutations affects the TEA-DNA binding domain, the first such example from any experimental organism.

Experimental Results

Phenotypes associated with the scalloped lethal alleles.

To gain a better understanding of the function and molecular properties of *sd*, five recessive lethal alleles that were generated in a screen for mutations uncovered by Df

(1)sd^{72b} and previously mapped to the C-terminal region of the SD protein, were further characterized (Campbell et al., 1991).

The mutations show different phenotypes and can be grouped in two categories based on the stage of lethality associated with the *sd* allele (Figure 5.1 A) and on the relative ability of the lethal allele to complement the wing phenotype of a weak *sd* allele, sd^{ETX4} (Figure 5.1 B). The sd^{3L} and sd^{47M} alleles belong to the early lethal class because they die in the first larval instar. The sd^{31H}, sd^{68L} and sd^{11L} comprise the late lethal class and these mutants survive through the larval instars and die at the pupal stage. Flies that are homozygous or hemizygous for these alleles also show gross head defects as previously reported (Campbell et al., 1991). In addition to recessive lethal phenotypes, sd^{3L}, sd^{47M}, sd^{68L} and sd^{31H} alleles display a *scalloped* wing phenotype when heterozygous with a weak allele of *sd*, sd^{ETX4} (Figure 5.2 A-F). That is, they do not complement the wing phenotype of sd^{ETX4}. In contrast, sd^{11L} gives a wild type wing when heterozygous with sd^{ETX4} (data not shown) that is indistinguishable from the wild type wing shown in Figure 5.2 A. Another notable feature of the heterozygous combinations of the lethal alleles with sd^{ETX4} is the varying wing sizes and degree of notching. The sd^{3L}, sd^{47M} and sd^{31H} alleles exhibit an extreme wing phenotype over sd^{ETX4} (Figure 5.2 C-E) that involves loss of majority of the wing blade tissue and erosion of the margin. In contrast, sd^{68L} when heterozygous with sd^{ETX4} shows very little removal of the wing blade tissue (Figure 5.2 F). However, variable erosion of the margin is seen ranging from very conspicuous to loss of only a few margin-specific bristles. Heterozygous $sd^{31H}/y^{+}Binsn$ females also show a dominant wing notching phenotype with a frequency of ~20% (8

out of 39 heterozygous females) and this combination has also been reported to exhibit ectopic bristles (Campbell et al., 1991).

The *sd* locus produces multiple transcripts as a result of alternative splicing events (Campbell et al., 1991). The E21 isoform is the best studied so far. However another isoform, E7, could potentially produce a different protein that would retain part of the TEA domain but differ extensively in the C-terminal half (Figure1 C). The lethal alleles were originally mapped by denaturing gradient gel electrophoretic analysis to the 3'end of the *sd* locus where the E21 and E7 isoforms differ. Because the lethal alleles can be grouped into two phenotypic categories, it was hypothesized that the wing complementing and wing non-complementing alleles could be a result of distinct mutations in the two alternative isoforms. Alternatively, the early and late lethal classes could be due to mutations in the various isoforms. To assess these two possibilities, the alleles were characterized by PCR amplification of the regions bounded by primers in Figure 5.1 C followed by sequencing of the amplified products.

Molecular lesions associated with the lethal alleles.

The molecular lesions associated with the different *sd* lethal alleles are described below and appear to affect only the protein encoded by the E21 isoform. The characterized lesions were superimposed on the E21 amino acid sequence and are presented in Figure 5.3.

***sd*^{3L}:** This allele is associated with a T→ A substitution resulting in a codon change of TTA→ TAA. This introduces a stop codon in place of a leucine at amino acid position 233 in the E21 isoform.

***sd*^{47M}**: In a previous study this allele was shown to be associated with a deletion of approximately 100bp located between an *Eco*R1 site at +12.2 and a *Bgl*II site at +12.6 of the genomic walk described in Campbell et al.(1991). Herein, it was found that this deletion is actually 157 base pairs in size and it removes the majority of intron 8 and also 22 nucleotides from exon 9. The result is the removal of seven amino acids as well as the splice site at the intron8/exon9 boundary, possibly giving rise to a protein that is defective beyond this point.

***sd*^{68L}**: This allele is associated with a T→ A substitution producing a codon change of TAC→ AAC. This introduces an asparagine in place of a tyrosine at amino acid position 326 in the E21 isoform.

***sd*^{31H}**: This allele is associated with a G→A mutation resulting in a codon change of AGA→AAA which causes an arginine to lysine substitution at amino acid position 144 of the E21 isoform.

***sd*^{11L}**: This allele is associated with an A→ T mutation resulting in a codon change of CAC→ CTC which causes a histidine to leucine substitution at amino acid position 433 of the E21 isoform.

As indicated, none of the above lesions are predicted to affect the E7 isoform.

Wild type residues mutated in sd lethal alleles are evolutionarily conserved.

If the lesions associated with the lethal alleles are affecting the structural integrity and thereby important functions of the protein, then the wild type residues are likely to be evolutionarily conserved. Thus, the amino acid positions mutated in the lethal alleles were examined and compared across phyla. The SD amino acid sequences from various organisms were aligned using the ClustalW software and the results are shown in Figure

5.4. The alignment data indicate that the encoded amino acids altered by mutations in various lethal alleles are conserved across different phyla which, in turn, implies that these amino acid positions are likely very important with respect to the structural and functional integrity of the protein.

The sd lethal alleles affect the TEA *domain and define a vestigial binding domain (VBD).*

As described above, most of the lethal alleles do not complement the wing phenotype of the sd^{ETX4} allele. However, sd^{11L} does complement the sd^{ETX4} wing phenotype resulting in normal wings. The molecular lesions associated with all but the sd^{31H} allele appear to be grouped together in the C-terminal region of the SD protein (Figure 5.3). The sd^{31H} allele affects the TEA domain. The molecular lesion associated with the sd^{11L} allele is the most C-terminal of all the alleles and is spatially somewhat removed from the other alleles in the C-terminal domain as shown in Figure 5.3. Based on the data, one can hypothesize that the wing non-complementing alleles, other than sd^{31H}, possibly affect a domain responsible for some function involved in wing development.

Using *in vitro* binding experiments, it was shown that the VG protein binds to the C-terminal region of the SD E21 isoform (Simmonds et al., 1998). In another study Vaudin et al., (1999) identified a domain in TEF-1 which binds a human *vestigial* orthologue TONDU. Protein sequence comparisons of the TONDU binding domain in TEF-1 with the SD sequence led to the prediction of a domain in SD that could bind VG. Furthermore, this domain in SD that bound VG was already known to lie in the C-terminal half of the protein (Simmonds et al., 1998). In light of the above evidence, and the fact that the wing non-complementing lethal alleles of *sd* lie in the C-terminal half of

the protein, where they appear to overlap with the above predicted domain, it is likely that the lesions associated with the sd^{3l}, sd^{47M} and sd^{68L} wing non-complementing alleles define the molecular boundaries of a domain responsible for binding VG *in vivo*. Thus, the effect of compromising this function is a wing non-complementing phenotype when heterozygous with sd^{ETX4}.

Localization of VG *in the wing discs of larvae harbouring various sd lethal alleles.*

VG is a nuclear protein that has no nuclear localization signal. Further, a VG protein lacking the SD interaction domain fails to get into the nucleus, suggesting that the binding of VG to SD is necessary for VG to get into the nucleus (Simmonds et al., 1998). More recently, it was shown that VG uses a putative NLS contained within the SD TEA domain to get into the nucleus (Srivastava et al., 2002). To test the claim that three out of the four wing non complementing alleles may have lesions that affect the VBD, VG localization was examined in the mutant wing discs from larvae harbouring the *sd* pupal lethal alleles. Because *sd* is on the X chromosome, 50% of the male larvae from the relevant crosses would be mutant and easily recoverable. Because the chromosome carrying the lethal alleles are also marked with the *yellow* (*y*) mutation it is easier to pick out mutant male larvae (y^-) from a population of y^+ animals. As previously mentioned, the non-complementing sd^{68L} allele affects the VBD while the complementing sd^{111L} allele does not. Because SD binding with VG is important for the nuclear localization of VG, it can be predicted that VG would be predominantly in the cytoplasm in sd^{68L} wing discs and would be nuclear in sd^{111L} wing discs. The data are in agreement with this prediction and, indeed, in discs from larvae carrying the sd^{68L} allele the VG localization is diffuse and predominantly cytoplasmic. In addition, the VG protein is localized to a narrow

domain within the wing pouch. This is in agreement with the VG localization observed using other viable hypomorphic *sd* alleles like sd^{58}, and is indicative of a compromise in the auto regulatory loop between SD and VG that helps maintain the gene product levels in the wing pouch (Chapter 3). The data for VG localization are shown in Figure 5.5 D-E". However, there is some variability in this localization in the sense that in some discs the VG protein appears very diffuse and cytoplasmic while in the others it appears that some VG protein could be in the nucleus. Sequence analysis of the sd^{11L} allele indicates that the lesion would not be predicted to affect the VBD. Thus, not surprisingly, VG localization in discs from larvae harbouring this allele is punctate and nuclear in nature. This is comparable to VG localization in wild type discs (compare Figure 5.5 C" to the wild type VG localization in 5.5 A" and to VG localization in sd^{68L}, 5.5 D" and 5.5 E"). Further support for the VBD being affected in *sd* wing non-complementing lethal alleles comes from the fact that mitotic clones of the sd^{47M} allele in wing discs exhibit diffuse localization of VG (Halder et al., 1998) in accordance with the proposal that the lesion in this allele affects the VBD.

The lesion associated with the sd^{31H} allele affects the TEA DNA binding domain and it also appears to be very close to a previously reported (chapter 2) Nuclear Localization Signal (NLS) (Srivastava et al., 2002), as well as a putative phosphorylation site (Underlined in Figure 5.3). Thus, it is possible that this mutation also affects the NLS. It has been reported that this NLS is utilized by VG to get into the nucleus (Srivastava et al., 2002) by virtue of the ability of VG to bind to SD. To test whether the sd^{31H} mutation affects the NLS, the nuclear localization of VG was examined in discs derived from sd^{31H}. If this lesion affects some aspect of the nuclear localization signal,

then VG in sd^{31H} derived wing discs should be diffuse and cytoplasmic. Surprisingly, there is no detectable VG within the wing pouch of these discs. However, the VG protein is seen in the hinge and notum areas (Figure 5.5 B-B"). In addition, the wing pouch also appears to be reduced in size.

Discussion

The data presented herein are relevant to several aspects of *sd* gene function. By analyzing the lethal alleles, an *in vivo* identification of a VBD for SD is determined. It is also shown for the first time that a mutation within the TEA DNA binding domain is both important for wing development as well as for viability of the fly. Finally, a possible molecular explanation for the observed variability in the transheterozygous wing phenotype of lethal alleles with a weak allele of *sd* is discussed below.

Identification of a VBD *in vivo*.

From *in vitro* studies it had been previously reported that the C-terminal half of SD bound to VG (Simmonds et al., 1998). However, it had not been shown if this was also true *in vivo*. The present analysis links mutations in the C-terminal half of SD to a domain responsible for binding VG. Of the five lethal alleles studied only four affect the wing phenotype and the physical lesions associated with three out of these four fall within the C-terminal half of SD and are localized within amino acids 233 to 326. These three mutations (sd^{3L}, sd^{47M} and sd^{68L}) define a VBD and this domain overlaps with a domain previously predicted by *in vitro* experiments to be responsible for binding VG (Vaudin et al., 1999). The sd^{3L} and sd^{47M} lesions are within the predicted domain and due to the molecular nature of these lesions would abolish the VBD completely. This suggestion is supported by the observation that the wing phenotype produced from these

alleles when heterozygous with sd^{ETX4} is more severe than the sd^{68L}/sd^{ETX4} phenotype and is indicative of reduced *sd* function (Figure 5.2 C-E compared to F).

The sd^{68L} lesion, on the other hand, is located just outside and 3' to the predicted VBD (Figure 5.3). Furthermore, the sd^{68L} allele is associated with the mutation of tyrosine, an amino acid that is subject to phosphorylation and dephosphorylation. Interaction between different proteins has been shown to be mediated by a phosphorylation-based mechanism and has been reviewed for a particular group of proteins called STATs (Darnell, 1997). Some of the wings from sd^{68L}/sd^{ETX4} flies appear to be almost wild type, with only a few margin-specific bristles missing. It is possible that this could be due to the variability of phenotype observed with the sd^{ETX4} allele. To minimize this problem, the wing phenotype of sd^{68L} in trans with the more severe but phenotypically stable sd^{58} allele was also examined. The wing phenotype of sd^{68L}/sd^{58} flies is also less severe than that produced by sd^{3L} or sd^{47M} over sd^{ETX4}. Thus, it appears that the sd^{68L} allele provides some wild type function in a genetic background shared with the sd^{ETX4} or sd^{58} alleles, so that the resulting wings are less severely mutant than sd^{ETX4} and sd^{58} homozygous wing phenotypes. It is possible that the protein produced from the sd^{68L} allele does not directly affect the VBD but instead affects the proper folding of the domain. Alternatively, since the limits of the VBD are not precisely defined it is also possible that the mutation of tyrosine in this allele is actually within the VBD and may be important for the regulation of binding VG via phosphorylation and dephosphorylation of this amino acid. However, this binding could be inefficient and result in the observed variability of the wing complementation phenotype with this (sd^{68L}) allele. Either way, sd^{68L} along with sd^{3L} and sd^{47M}, do affect the VBD. Indirect support for this also comes from locating the molecular

lesion associated with the sd^{11L} wing phenotype complementing allele. A likely reason for this complementation could be that the lesion in this allele does not affect the VBD because it is located more distally (78 amino acids from the sd^{68L} mutation towards the C-terminal end of SD) (Figure 5.3). Transvection (Duncan, 2002) was also considered as a possible explanation for the observed complementation between sd^{11L} and sd^{ETX4}. According to current transvection models, a wild type enhancer from sd^{11L} would be able to drive expression of the *sd* gene from a sd^{ETX4} homologue in a pairing-dependent manner. It is known that inversions will block the pairing of chromosomes in trans and thus block transvection. To test this hypothesis, complementation between the sd^{11L} and sd^{58} (an allele, associated with a large inversion that would disrupt any pairing mediated complementation) alleles was examined. The data show that complementation is observed even when sd^{11L} allele is in trans with sd^{58}, thus arguing against transvection as an explanation for the complementation observed. Support for the mutations in sd^{3L}, sd^{47M} and sd^{68L} affecting the VBD and sd^{11L} not affecting this domain also comes from VG localization data in wing discs derived from sd^{68L} and sd^{11L} hemizygous larvae (Figure 5.5 C-E") as well as from VG localization in mitotic clones of the sd^{47M} allele. As shown, VG in sd^{68L} wing discs (Figure 5.5) and in *sd* mutant clones harbouring the sd^{47M} allele is diffuse (Halder et al., 1998) rather than nuclear. This is a clear indication that the VBD encoded by these alleles is impaired, reinforcing the previous findings that binding of SD to VG is important for the nuclear localization of VG. However, VG in sd^{11L} wing discs is nuclear, thereby supporting the claim that in this allele the encoded VBD is unaffected.

Mutation in the *sd* TEA DNA binding domain affects viability and wing development.

To date, our knowledge about the TEA DNA binding domain has been based primarily on *in vitro* mutational analysis. However, extrapolation from *in vitro* observations to *in vivo* functions is not always valid. Herein, the correlation between a mutation within the conserved TEA DNA binding domain and its effects on specific phenotypes *in vivo* is reported for the first time. The TEA DNA binding domain has been previously predicted to have three helices. However, the limits of the third helix within the domain are not very well defined (Burglin, 1991). The mutation associated with the sd^{31H} allele (Arg to Lys) appears to affect the TEA DNA binding domain between the second and third helices (Figure 5.3). This lesion also appears to lie between two putative phosphorylation sites, and the role of phosphorylation in regulation of DNA binding by the TEA domain from organisms other than Drosophila is well documented (Gupta et al., 2000; Jiang et al., 2001). The cause of the observed heterozygous wing and homozygous lethal phenotypes associated with this allele can be explained in a number of ways. The phenotypes could simply be the result of a defect induced by this mutation in regulation of DNA binding by phosphorylation. The second and the third helices of the TEA DNA binding domain have been suggested to be the domains that contact DNA (Burglin, 1991). This mutation could also affect directly the ability of the second and third helices within the TEA domain to contact DNA. Thus, the mutation would result in inefficient to no contact with DNA by the TEA domain, thereby preventing transcription of genes controlled by *sd*. It is also possible that the mutation in sd^{31H} affects the nuclear localization signal found within the TEA DNA binding domain of SD, so that SD is

prevented from entering the nucleus. In the absence of a SD antibody, it is difficult to determine if the mutation prevents SD from entering the nucleus or simply results in inefficient transport of the protein to the nucleus. The late pupal lethality associated with this allele is consistent with an argument that this mutation results in inefficient transport of SD to the nucleus. The mutant animal is able to survive until the pupal stage, beyond which the level of SD in the nucleus would be unable to sustain the level of transcription needed for survival. However, the VG localization data from sd^{31H} mutant discs argue against a defect in nuclear localization of SD. Because SD is needed for maintenance of *vg* and *sd* expression, one would see some VG in the wing pouch of the mutant discs if the mutation was causing inefficient nuclear localization of SD. The absence of any noticeable VG in the wing pouch (Figure 5.5 B-B") favours the hypothesis that the mutation affects the DNA binding ability of the TEA domain, so that this mutant SD cannot bind DNA elements and as a result VG expression in the wing pouch is possibly not maintained.

Model to explain the variability in the complementation of the *sd* wing phenotype.

To explain the variability in the heterozygous wing phenotype between sd^{ETX4} and the different lethal alleles, a model is presented in Figure 5.6 where it is assumed that equal amounts of VG and SD are needed for normal wing development. The binding of SD to VG enables the translocation of VG into the nucleus where the complex binds to the target DNA motifs and activates transcription of the downstream target genes (Figure 5.6 A). In sd^{ETX4}, a hypomorph, there are fewer molecules of SD produced due to a mutation in the regulatory region, so that the number of functional SD/VG complexes is reduced. This, in turn, could result in a decrease in transcription of downstream genes

manifesting itself in a mild *sd* mutant wing phenotype (Figure 5.6 B). The sd^{58d} allele is a more severe hypomorph so that even fewer molecules of SD are produced (compared to sd^{ETX4}) resulting in an even more compromised production of downstream gene products (Figure 5.6 C). Thus, the resulting wing phenotype is more severe than that produced by sd^{ETX4}. Heterozygotes between sd^{ETX4} and sd^{58d} display a wing phenotype that is intermediate between that produced by either allele as a homozygote. A possible scenario leading to the production of the intermediate phenotype is depicted in Figure 5.6 D. In this heterozygous combination the amount of functional SD/VG complex could be intermediate between either sd^{ETX4} or sd^{58} alone, resulting in an intermediate phenotype. The wing phenotype of sd^{ETX4}/sd^{3L} or sd^{47M} flies is similar to sd^{58d}. A possible molecular explanation for this is depicted in Figure 5.6 E. In the above heterozygous combinations, the sd^{ETX4} allele possibly produces fewer SD molecules than a wild type gene while the sd^{3L} or sd^{47M} alleles produce wild type levels of SD molecules that are defective in binding VG. Thus, in effect, it is possible that the amount of functional SD/VG complex is comparable to the levels in homozygous sd^{58} individuals, resulting in decreased transcription from the enhancers in this combination and hence the phenotype. Further, the sd^{31H} allele would permit a level of SD production comparable to the amount from a wild type allele. However, due to the nature of the mutation in this allele the protein would possibly be defective in binding DNA (Figure 5.6 F). Thus, the sd^{31H}/sd^{ETX4} combination would also result in fewer functional SD/VG molecules (comparable to the amount of functional SD/VG complexes in sd^{58d} individuals) and a decreased level of transcription from the enhancers manifested in a wing phenotype resembling that of sd^{58d} flies.

In this chapter the molecular characterization of lethal alleles of *sd* is reported and this analysis enables one to associate specific residues within the mutated proteins to the mutant phenotypes. The analysis also defines a VBD in SD. This is the first report that identifies specific amino acid residues in SD which appear to be fundamentally important for the function of the protein. Because the residues affected in the *sd* lethal alleles are conserved across species and phyla, this study could also have important implications in understanding the properties of the vertebrate homologue TEF-1.

Materials and Methods

Drosophila melanogaster stocks and crosses.

The genotypes of the *sd* stocks used in this study are described in detail in Campbell et al., (1991, 1992) and were obtained from Shelagh Campbell. The five recessive lethal *sd* alleles were tagged with *yellow* (y^-) using standard genetic techniques and the y^--tagged alleles were maintained as balanced stocks over a *FM7* or a *FM6* balancer chromosome that also carries a y^- marker. The y^--tagged females were crossed to y^+*BinSn* males and y^+ single bar females were selected and then re-crossed to y^-*BinSn* males. The hemizygous y^- male larvae carrying the *sd* lethal lesion were separated from their y^+ sibs by visual examination of mouth hooks under a dissecting microscope. The sd^{ETX4} enhancer trap used has been described in detail (Campbell et al., 1992). The y^+*BinSn* stock was obtained from the Drosophila Stock Center at Bloomington, Indiana, USA.

Genomic DNA isolation.

Approximately 10-20 *yellow* first instar larvae from each *sd* larval lethal stock and approximately 10 yellow third instar larvae from the pupal lethals were used to isolate

genomic DNA using a home made glass milk-based DNA extraction protocol. In brief, genomic DNA was extracted with phenol and chloroform and incubated with glass milk. The glass milk-treated DNA was eluted in 30µl of distilled water. For PCR, 2µl of the DNA solution obtained was used in each reaction.

PCR amplification of Scalloped regions.

For PCR amplification, Platinum PCR supermix from GIBCO BRL was used in a reaction containing 2 µl of genomic DNA (isolated above) from each *sd* allele and 5 ng of each primer. The sequences of PCR primers used for amplification of the different regions of *sd* are shown below and their positions are depicted in Figure 5.1 C. These primers span most of the coding region including the N-terminal TEA domain. The first three exons were not sequenced as these *sd* alleles were previously mapped to the C-terminal half of the *sd* locus (Campbell et al., 1991).

Primer designation	**Sequence, 5' to 3'**	**Tm** degC
#1	CCAGGATCCCACCACCCATCACATATACC	73.4
#2	GCGAATTCGCTCTGATTGTGTCGTTCCTTG C	77.2
#3	CCAGGATCCCAATTCTGGCAACCTGGACTA CAGC	78.8
#4	GCGAATTCACGGTATATGAGATGGGTGGT GC	75.2
#5	CCAGGATCCGGCATACGTCAGTTCTTAGAT GTGTGC	78.3
#6	GCGAATTCCATGCTCATCCAACCCAAGGAT CTAGC	78.8

The PCR reactions were performed on a Perkin Elmer thermal cycler using the following amplification cycles: 95°C for 5 minutes, followed by 40 cycles at 94°C for 1.0

minute, 60°C for 1.0 minute and 72°C for 3.0 minutes. This was followed by a hold of one cycle at 72°C for 7 minutes and then storage at 4°C.

Sequencing of the amplified products.

The amplified product was either sequenced directly or cloned into pGEMT vector from Promega and then sequenced using an Amersham Dyenamic ET kit according to the manufacturer's instructions. For products that were cloned, multiple independent clones were sequenced to detect possible errors incorporated during cloning manipulations. Once a particular putative change was identified it was confirmed by sequencing DNA amplified from a heterozygote. The sequencing reactions were run by Pat Murray and Lisa Ostafychuk of the MBSU.

Sequence analysis.

All sequence analysis was done using DNAMAN sequence analysis software by Lynnon BioSoft, Montreal Canada. The assembled sequence was aligned against wild type sequence in the database using the blast server at http://www.ncbi.nlm.nih.gov/blast.

Immunohistochemistry.

All immunohistochemical staining was done according to a published protocol (Williams et al., 1991). The anti-VG antibody was a gift from Sean Carroll, University of Wisconsin, and has been previously described (Williams et al., 1991). The secondary antibody was a Cy3 conjugated anti-rabbit antibody (Jackson ImmunoResearch).

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Figure 5.1. Characterization of *sd* lethal alleles and phenotypic classification. A) Classification of *sd* lethal alleles according to the stage of lethality and B) The ability to complement the wing phenotype of a weak allele of *scalloped*, sd^{ETX4}. C) PCR primers used to amplify regions bounded by each pair, and collectively, covering the 3' exons of the locus. The gene structure corresponding to two alternatively spliced isoforms is shown (Campbell et al., 1991). Filled boxes represent exons and lines represent introns. Of the two isoforms shown, *sd* E21 is the best studied. Exons 1-3 were not included in this study as the lethal alleles had been mapped in an earlier report to the C-terminal half of the *sd* locus using denaturing gradient gel eletrophoresis (Campbell et al., 1991).

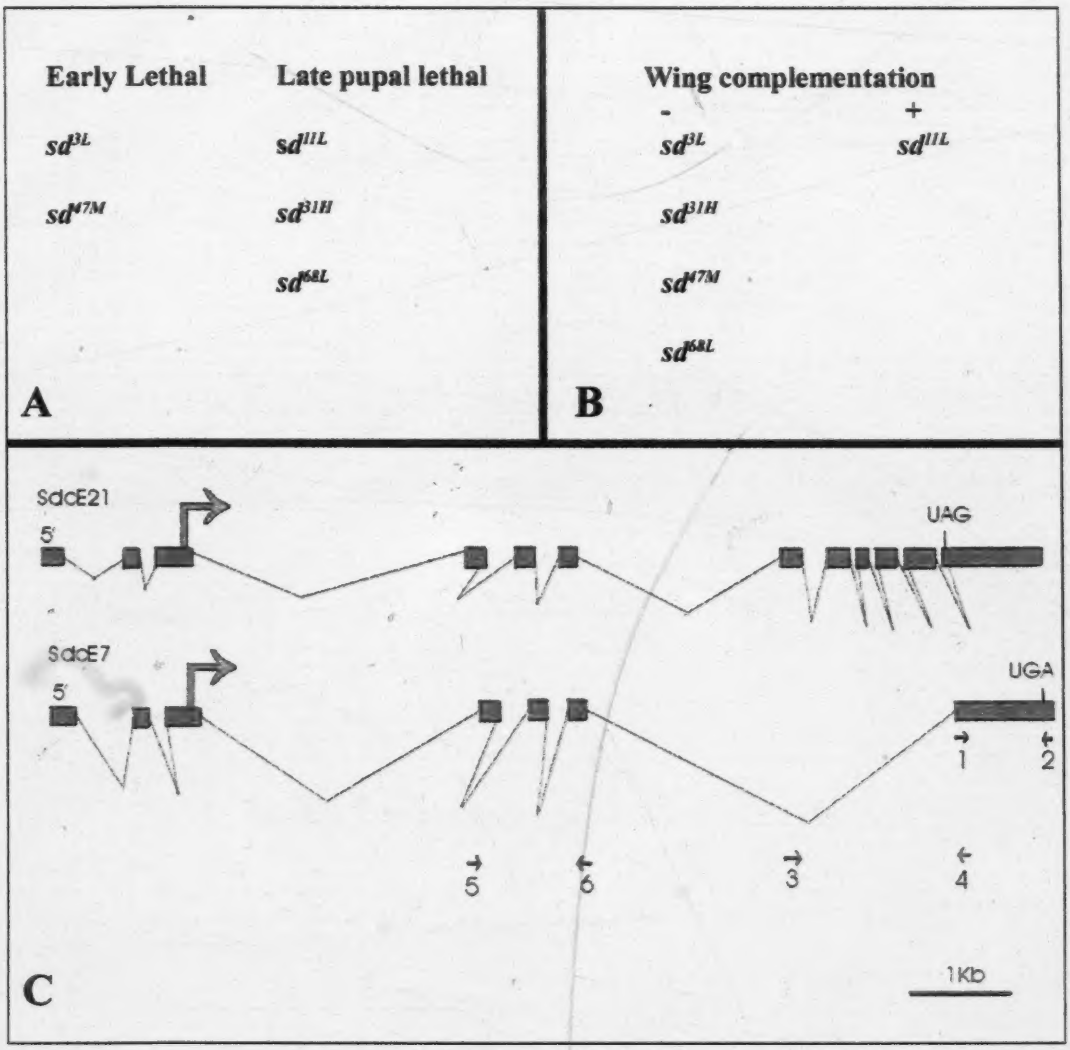

Early Lethal
Late pupal lethal
sd^{3L}
sd^{M7M}
$sd^{III L}$
sd^{31H}
sd^{68L}
A
Wing complementation
-
+
sd^{3L}
$sd^{III L}$
sd^{31H}
sd^{M7M}
sd^{68L}
B
SdcE21
5'
UAG
SdcE7
5'
UGA
1
2
5
6
3
4
1Kb
C

Figure 5.2. The heterozygous wing-phenotype of recessive lethal and viable *sd* alleles. A-H) Whole mount of wings from various *sd* allelic combinations. A) Wild type wing showing an intact wing margin. B) Wing from a sd^{ETX4} female showing the notches in the wings. C-F) Wings from heterozygotes between the *sd* lethal allele indicated and sd^{ETX4}. Note the variability in the wing phenotype in the different heterozygous combinations. G) Wing from a sd^{58} fly showing a more extreme wing phenotype compared to the sd^{ETX4} phenotype. H) Wing from a heterozygote between sd^{ETX4} and sd^{58d}. Note that the phenotype is intermediate between a sd^{ETX4} and sd^{58d} wing phenotype. The sd^{11L}/sd^{ETX4} combination is not shown, as in this combination the wings are indistinguishable from the wild type wing shown in A.

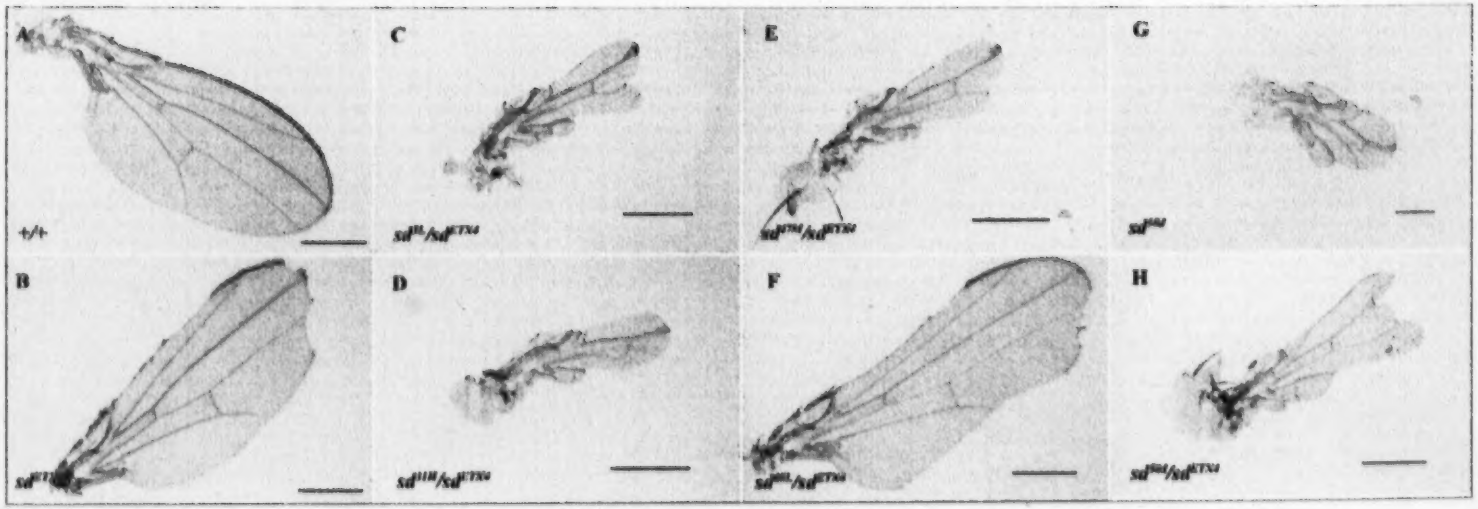

A
+/+
C
sd^{10}/sd^{ETX4}
E
sd^{ETX4}/sd^{ETX4}
G
sd^{404}
B
sd^{ETX4}
D
sd^{1118}/sd^{ETX4}
F
sd^{1002}/sd^{ETX4}
H
sd^{504}/sd^{ETX4}

Figure 5.3. Molecular lesions associated with the *sd* lethal alleles. The specific lesions are superimposed over the wild type SD E21 isoform sequence (Campbell et al., 1992). The wild type amino acid associated with the various lethal alleles is depicted with an oversized letter. The lethal allele designation is indicated above the affected amino acid. The mutated amino acid substitutions are shown over the wild type sequence for the various alleles. The coloured boxes represent known and predicted domains in the SD protein. The Blue box corresponds to the TEA DNA binding domain (Burglin, 1991; Campbell et al., 1992) whereas a predicted VBD (Vaudin et al., 1999) is shown as a magenta box. Various helices in the DNA binding domain are marked by a line over the respective amino acids. The nuclear localization signal within the TEA domain is depicted by a red box. A putative protein phosphorylation site is marked with a red line under the respective amino acids. The encoded amino acids deleted in the sd^{47M} allele are shown with a black bar over the affected amino acids. While sd^{3L} and sd^{47M} affect the predicted VBD, sd^{31H} affects the TEA DNA binding domain and sd^{111L} does not affect either domain. This study extends the 3' limit of the VBD. The extension is indicated by a brown line under the corresponding amino acid sequence and includes the sd^{68L} allele.

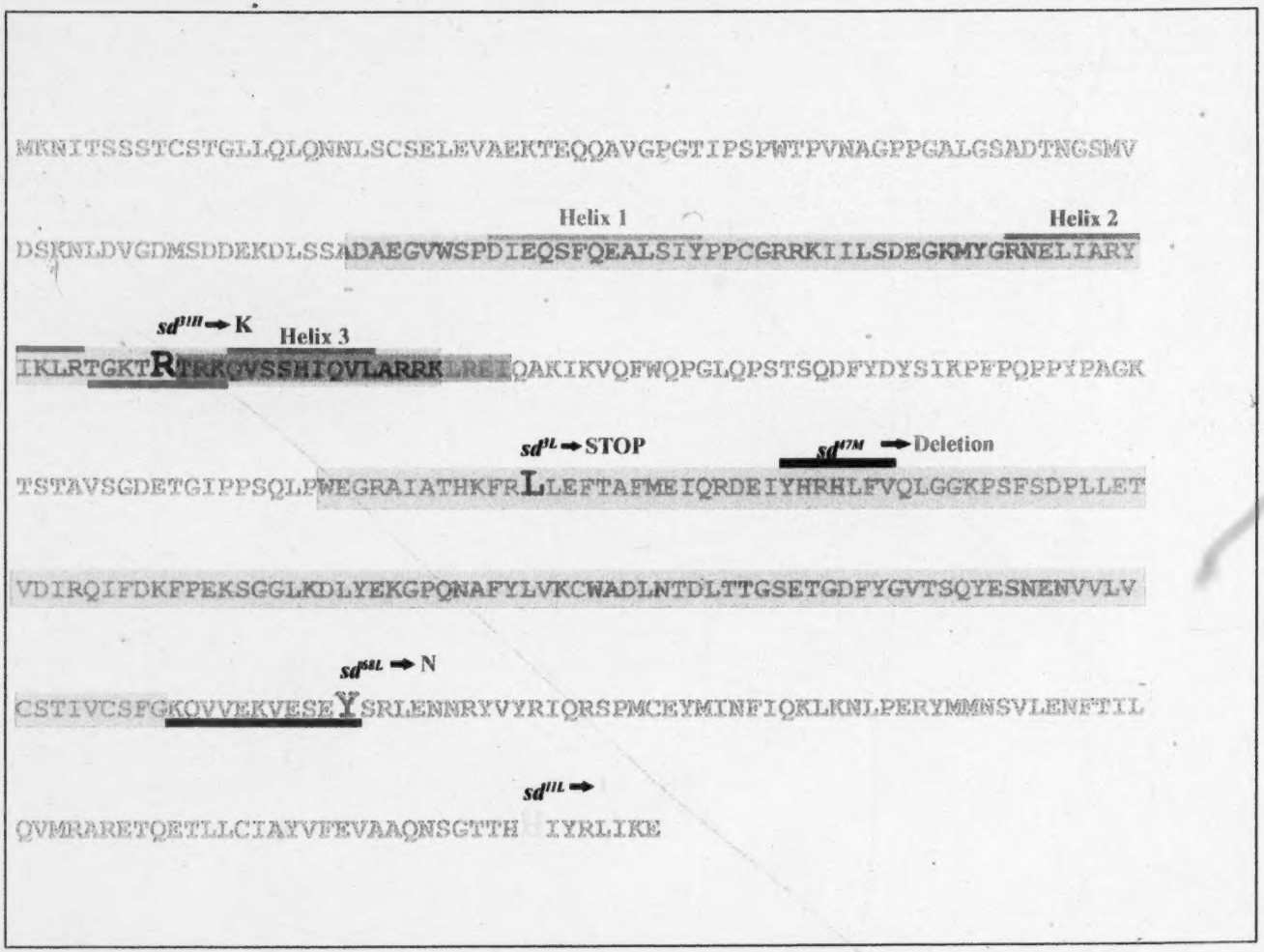

MKNITSSSTCSTGLLQLQNNLSCSELEVAEKTEQQAVGPGTIPSPWTPVNAGPPGALGSADTNGSMV
Helix 1
Helix 2
DSKNLDVGDMSDDEKDLSSADAEGVWSPDIEQSFQEALSIYPPCGRRKIILSDEGKMYGRNELIARY
sd^P1H → K
Helix 3
IKLRTGKTRTRKGVSSHIQVLARRKLRETQAKIKVQFWQPGLQPSTSQDFYDYSIKPFPQPPYPAGK
sd^P1L → STOP
sd^47M → Deletion
TSTAVSGDETGIPPSQLPWEGRAIATHKFRLLEFTAFMEIQRDEIYHRHLFVQLGGKPSFSDPLLET
VDIRQIFDKFPEKSGGLKDLYEKGPQNAFYLVKCWADLNTDLTTGSETGDFYGVTSQYESNENVVLV
sd^58L → N
CSTIVCSFGKQVVEKVESEYSRLENNRYVYRIQRSPMCEYMINFIQKLKNLPERYMMNSVLENFTIL
sd^111L →
QVMRARETQETLLCIAYVFEVAAQNSGTTH IYRLIKE

Figure 5.4. Evolutionary conservation of the residues affected by various *sd* lethal alleles. A-D) Portions of the wild type Drosophila SD sequence aligned with homologues from human TEF-1, mouse TEF-1, chicken TEF-5, *C. elegans egl-44* and Yeast TEC-1 showing the sequence identity in red and similarity in gray. The amino acid affected, in each lethal *sd* allele is marked by an arrow over the respective wild type amino acid. A) Wild type amino acid in sd^{31H} is mutated from an arginine to lysine, B) in sd^{3L} from a leucine to a stop codon, C) in sd^{68L} from a tyrosine to an asparagine, D) in sd^{11L} from a histidine to a leucine.

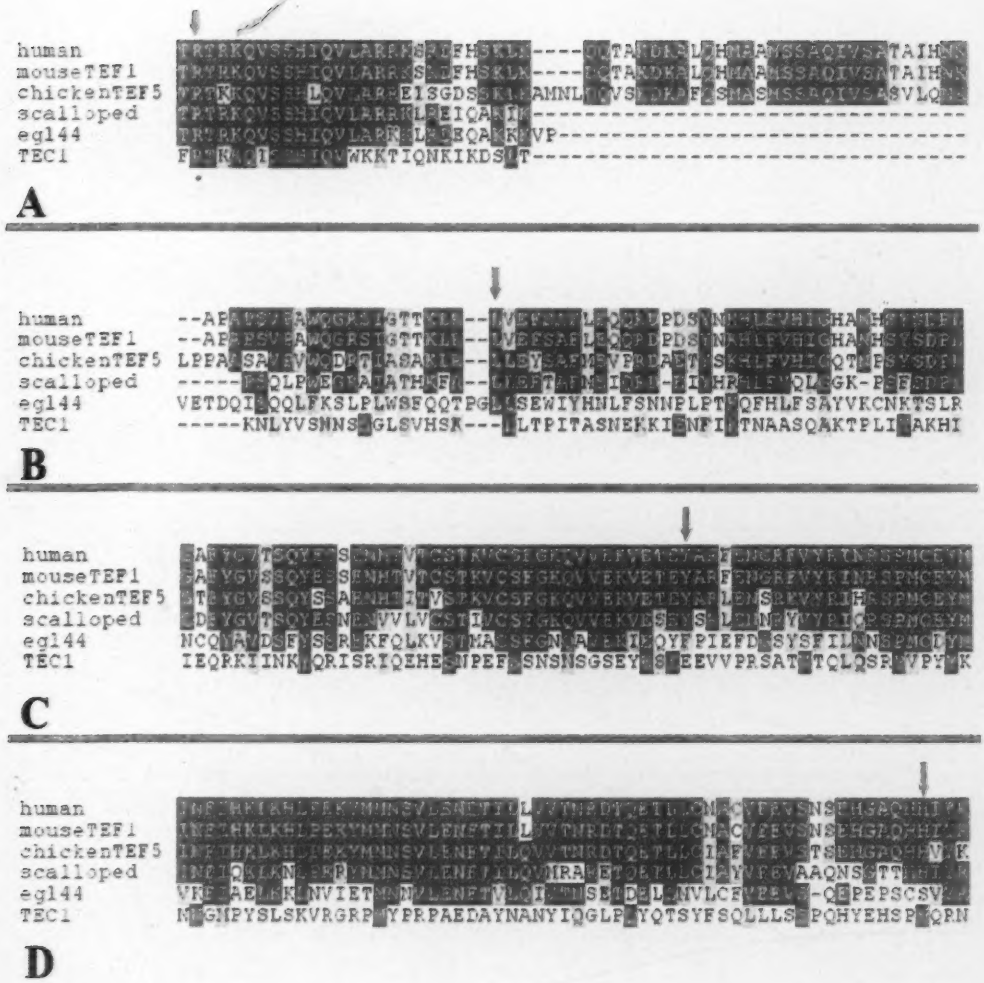

human
mouseTEF1
chickenTEF5
scalloped
egl44
TEC1
A
B
C
D

Figure 5.5. VG localization in wing discs from various pupal lethal mutant allelic backgrounds. The wing discs are derived from larvae harbouring various pupal lethal *sd* alleles and stained for VG protein (Red channel). Nuclei are stained with DAPI which gives off a blue fluorescence (marked with ' in all genotypes) and the merge between the two channels is shown with a ". A-A") Wild type wing disc exhibiting the nuclear localization of VG. B-B") Wing disc derived from a sd^{31H} hemizygous larva. This disc is at a different magnification from the others to highlight the fact that the hinge and notum expression of VG is not affected. Note the loss of any wing pouch-specific VG localization. C-C") Wing disc from a sd^{11L} hemizygous larva showing the nuclear localization of VG as judged by the punctate pattern and the magenta colour in the merge. D-D") Wing disc from a sd^{68L} hemizygous larva showing the diffuse and cytoplasmic localization of VG, and seen more clearly in the magnified regions shown in E-E".

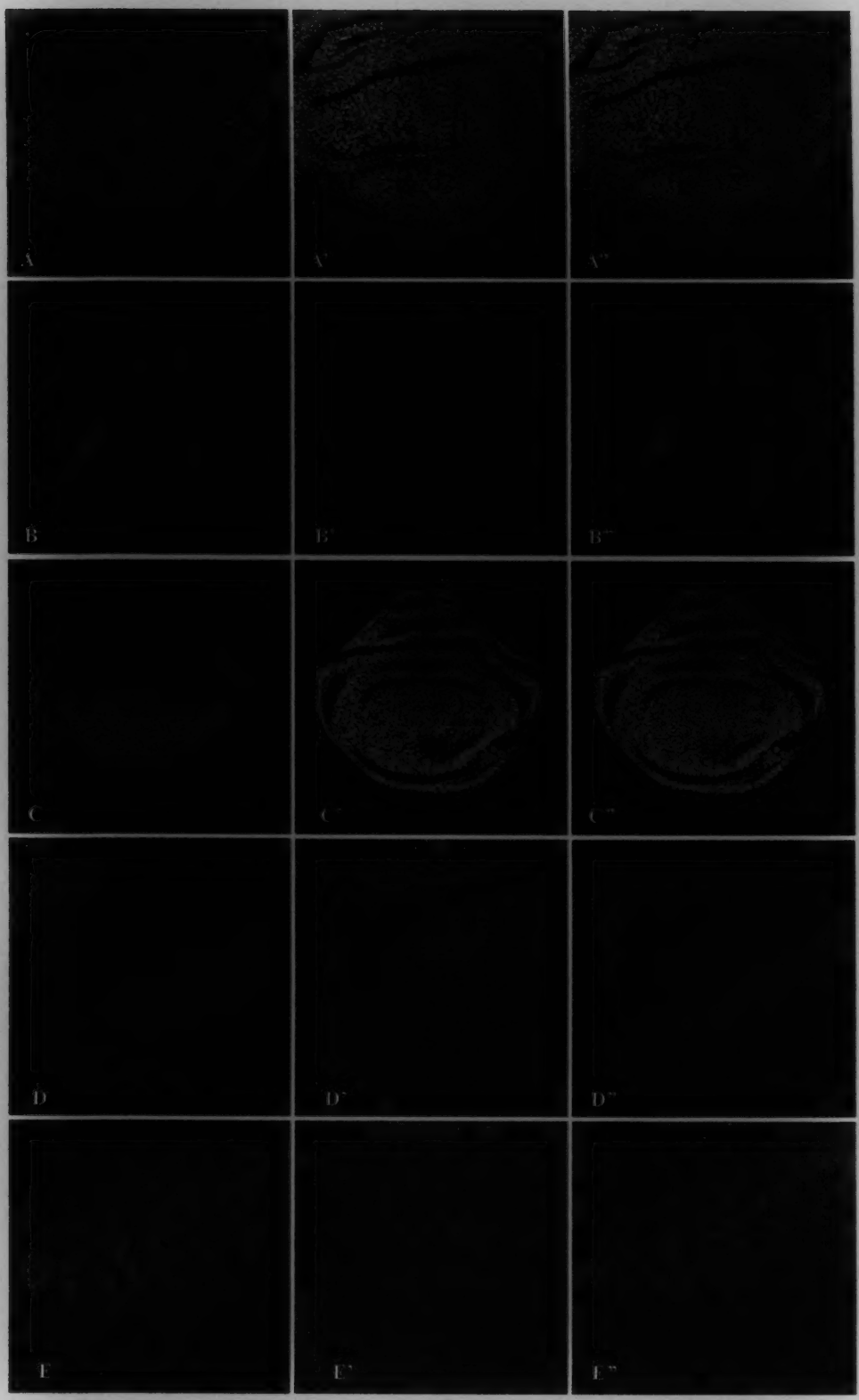
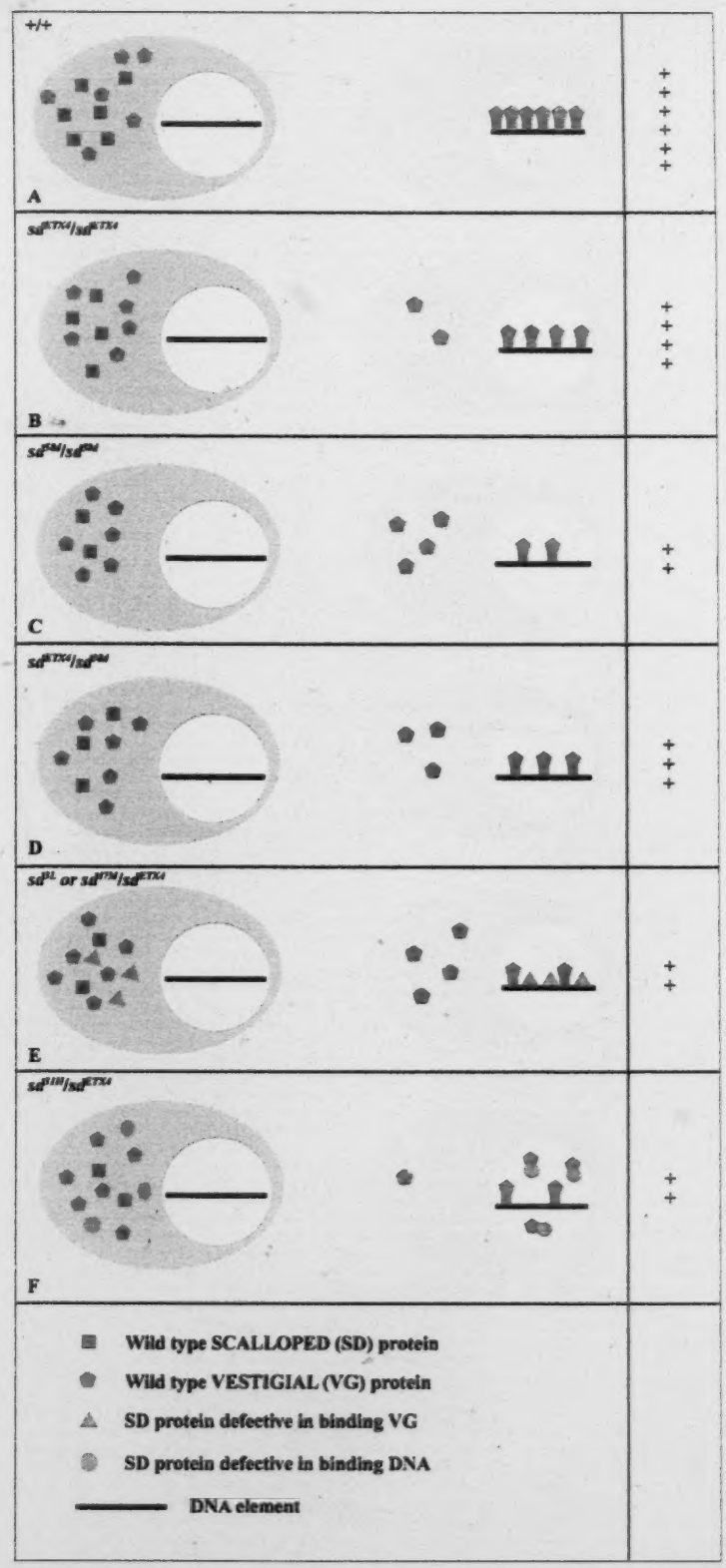

A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''

Figure 5.6. A model to explain the molecular events that result in the variability associated with different *sd* alleles heterozygous with sd^{ETX4}. The genotypes are indicated in the top left hand of panels. The + in the right hand column represents the transcriptional output from each genotypic combination. The outer circle represents a cell and the inner circle represents the nucleus, the space in between the cytoplasm. A) In wild type cells SD and VG are possibly present in equal amounts. Upon binding to each other the complex enters the nucleus facilitated by the NLS in the SD TEA domain. The complex would then bind to the enhancer elements resulting in expression of downstream target genes. B) In a sd^{ETX4} hypomorph, the level of SD is below the wild type level resulting in fewer SD/VG complexes, and thus a lower activation of target genes. C) The sd^{58d} allele is a more severe hypomorph producing even lower amounts of SD compared than sd^{ETX4}, and thus fewer SD/VG complexes resulting in even lower activation of target genes. D) In a sd^{ETX4}/sd^{58d} cell the amount of SD produced is intermediate between that produced by either sd^{ETX4} or sd^{58d} alone, resulting in an intermediate activation of downstream target genes and an intermediate wing phenotype. E) Due to the nature of the mutation in the sd^{3L} and sd^{47M} alleles, the protein produced would be one that would be defective in its ability to bind VG but which could still bind VG. Thus, the amount of functional SD/VG complexes available to activate downstream genes in sd^{3L}/sd^{ETX4} or sd^{47M}/sd^{ETX4} individuals would be as low as in sd^{58d} homozygotes, hence causing a severely defective wing phenotype. F) The sd^{3111} allele affects the TEA DNA binding domain and it is postulated that while the SD protein produced by this allele would be able to bind VG it would be defective for binding DNA. Thus, the amount of functional SD/VG complexes would be low and similar to the levels found in sd^{58d}.


+/+
A
sd^ETX4/sd^ETX4
B
sd^DM/sd^DM
C
sd^ETX4/sd^DM
D
sd^2L or sd^ITM/sd^ETX4
E
sd^110/sd^ETX4
F
Wild type SCALLOPED (SD) protein
Wild type VESTIGIAL (VG) protein
SD protein defective in binding VG
SD protein defective in binding DNA
DNA element

Chapter 6

In vivo analysis of functional domains of the *vestigial* gene. ¶

Introduction

The adult wing in *Drosophila* is made up of the wing hinge, wing blade and the mesonotum. Apart from its function in flight, the wing blade also serves a sensory function and is covered with sensory bristles all along the margin. The wings in *Drosophila* develop from a sac made up of a single layer of columnar epithelial cells called the wing disc (Cohen, 1993) that gives rise to the morphologically distinct hinge, blade and mesonotum of the adult. Recently, it has been proposed that separation of the wing pouch from the hinge in the wing disc might be happening by regulation of affinity of the cells and it is thought that *vestigial* (*vg*) possibly plays a role in this process (Liu et al., 2000). VESTIGIAL (VG) is a novel nuclear protein with no obvious homologies to any other known protein except within a 56 amino acid motif that has been demonstrated *in vitro* to be necessary and sufficient for binding SCALLOPED (SD) (Simmonds et al., 1998). The partnership of VG and SD in wing development is well documented (Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002). This 56 amino acid domain has been termed the SD interaction domain (SID) and is thought to be important in the formation of a functional VG/SD transcription complex (Halder et al., 1998; Simmonds et al., 1998). The SID has also been shown to be

¶ A version of this chapter has been published. MacKay et al., 2003 Genetics **163-4**: 1365-73

important for the nuclear localization of VG because when the *vg* gene is deleted for the SID region the result is a cytoplasmic accumulation of VG (Simmonds et al., 1998). In addition to the SID, VG has also been shown to have two additional functional domains that appear to mediate target gene activation from the VG/SD transcription complex. This classification was based on the inability of the activation domain deletions to rescue *vg* mutations and also on the relative ability of the deletions to induce *sd* gene expression when expressed ectopically (MacKay et al., 2003). In addition to the activation domains, an additional region in VG comprised of 91 amino acids has been reported to be dispensable in the above assays without compromising the overall function of VG. Based on the above *in vivo* study, different regions of VG important for some of its functions have been identified and are depicted in Figure 6.1.

While the authenticity of the various domains in VG has been confirmed *in vivo*, the functionality of the SID by its own has not yet been demonstrated *in vivo*. The involvement of this domain in nuclear localization of VG has, however, been demonstrated *in vivo* by using a *vg* construct carrying a deletion of this domain which results in cytoplasmic VG (Simmonds et al., 1998). Herein, the role of each of the activation domains in VG is investigated with respect to the role they might play in contributing to the abilitiy of VG to induce WINGLESS (WG) in a non-cell autonomous fashion. The role of these activation domains in the promotion of adhesive properties of cells in which they are expressed is also examined (Liu et al., 2000). In addition, a demonstration of the *in vivo* consequences of expressing the SID in the wings is presented. The SID can function *in vivo* just as it does *in vitro*. The generation of a

reagent that could be used to further our understanding of wing development by the SD/VG complex is also described.

Results and Discussion

Flp-out clonal analysis of VG activation domains.

Control Flp-out clones expressing just the green fluorescent protein (GFP) have irregular boundaries, suggesting that within these clones there are adhesive differences due to a gradient of gene expression within the wing pouch. In addition, these, control clones are unable to induce *wg* and fail to grow (Figure 6.2). However, Flp-out clonal analysis using a full-length *vg* construct has shown that *vg* plays a role in the promotion of adhesive properties in the cells that it is expressed in (Liu et al., 2000). This is exemplified by the production of larger clones with rounder smoother boundaries compared to control GFP clones. In addition to promoting adhesiveness, VG also promotes clonal growth and induction of WG in a non-cell autonomous manner in the hinge (Liu et al., 2000).

To understand if VG needs one or both activation domains for satisfying its above mentioned roles, deletion constructs encompassing different domains (Figure 6.3) were expressed in Flp-out clones (Ito et al., 1997; Papayannopoulos et al., 1998; Struhl et al., 1993). Deletion of either of the activation domains results in clones that fail to grow (smaller compared to wild type VG expressing clones), have an irregular shape (demonstrating the existence of adhesive differences within the clones) and are unable to induce WG in the clones that overlap the hinge region (Figure 6.4 A-F). Clones expressing a construct encoding either full length *vg* or another deletion construct that retains both activation domains, result in larger, circular clones that are capable of

expressing WG in the hinge in a non-cell autonomous manner (Figure 6.4 G-L). Note the localization of WG surrounding the clones in Figure 6.4 G-L marked by white arrowheads. These results imply that, while the individual activation domains might retain some residual activation properties, both are required for normal VG function. Thus, for formation of the wing and for satisfying the functions attributed to VG in this context, both the activation domains must be present.

The SID from VG can function *in vivo* independently of the activation domains.

As mentioned earlier, the SID was identified as a 56 amino acid domain based entirely on *in vitro* binding experiments (Simmonds et al., 1998) and was shown to be necessary and sufficient for binding SD. The *in vivo* importance of the SID in the nuclear localization of VG was also determined. To see if this domain on its own could function *in vivo*, the UAS/Gal4 (Brand and Perrimon, 1993) system was used to express a UAS construct encoding the SID but omitting the two activation domains. This construct was made by PCR amplification of the DNA corresponding to the VG sequence from amino acids 171-335, and is depicted in figure 6.5 B (also see Materials and Methods for details). We hypothesized that, if the SID is functional *in vivo*, over-expression of this in the wing disc should result in titration of the endogenous SD supply. This is because the truncated VG protein would be a competitor with normal VG for SD binding and this would result in a decrease in the functional SD/VG complex within the wing pouch, which should manifest itself in the wing as a phenotype similar to *sd* mutant wings. Thus, the truncated VG should cause a dominant negative effect manifested in a mutant phenotype. Indeed, expression of UAS-SID within the wing pouch by using a *vg*-Gal4 driver results in wings that resemble a *sd* mutant phenotype and display the characteristic

notching of the wing margin (Figure 6.5 D compared to C). This result clearly implies that the SID of this construct is functional *in vivo* and is capable of interacting with SD, thereby precluding normal VG from doing so. Recently, in vertebrates several genes having significant homology within the SID have been identified (Maeda et al., 2002; Mielcarek, 2002; Vaudin et al., 1999) and these genes are expressed in tissues where the vertebrate homologue of *sd,* TEF-1, is expressed. It has been shown that some of these proteins interact with TEF-1 via the conserved SID (Maeda et al., 2002; Vaudin et al., 1999). The generation of a dominant phenotype by expressing SID alone, as reported herein, offers an avenue for carrying out a suppression screen against the resulting dominant negative wing phenotype. It is possible that one would be able to identify new classes of genes involved in restoration of the margin bristles and also involved in proliferation.

Given its demonstrated ability to bind to SD, UAS-SID could also be used to titrate SD levels from specific tissues in order to understand the function of *sd* in other tissues. For example, *sd* is also known to be in expressed in eye, antennal, and the third leg discs (Campbell et al., 1992). The majority of the known mutations in *sd* either affect the wing or are lethal. To date, the failure of mitotic clones of *sd* null alleles to grow well has prevented any extensive clonal analysis. The ability to remove *sd* function in a tissue-specific controlled manner, owing to the temperature sensitivity of the UAS/Gal4 system (Speicher et al., 1994) that this UAS-SID construct offers, would be helpful in understanding the role of *sd* in tissues other than the wing.

Materials and Methods

Flp-out clones.

Flp-out clones (Ito et al., 1997; Papayannopooulos et al., 1998) were induced in flies heterozygous for an actin>y+>GAL4(AyGAL4)UAS-GFP chromosome (Y. Hiromi) and the relevant *vg* deletion-bearing UAS construct according to the protocol described in Liu et al.(2000). Respective larvae with the above genotype also carried a hs-*FLP122* construct (Struhl et al., 1993). Briefly, the clones were induced by heat shock and then allowed to grow for 48-72 hours at 25C after induction. Subsequently, the discs were dissected and stained with antibodies.

Antibody staining.

Antibody staining was performed as described in Williams et al., (1991). VG antibody was obtained from S. Carroll and used at 1:400 dilution. WG antibody was obtained from Developmental Studies Hybridoma Bank and was used at a dilution of 1:400.

Construction of UAS-*vg*SID transgenic flies.

The construct encoding a partial *vg* gene (amino acids 171-335) was made by PCR amplification of a region corresponding to the coding region for above amino acids and then cloning the product into a modified pUAST vector that also carries the Myc epitope tag for subsequent localization of the protein. The construct was injected in *yw* flies using a standard protocol and independent lines were established by Sandra O'Keefe and Monica Engstrom.

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Mielcarek, M.G., S. Krüger,M.Braun,T. (2002) VITO-1, a novel vestigial related protein is predominantly expressed in the skeletal muscle lineage. Gene Expression Patterns 2, 305-310.

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Figure 6.1. Domain structure of VG.

The various VG domains identified either *in vitro* or *in vivo* are depicted. AD1 and AD2 refer to the N-terminal Activation domain and C-terminal Activation domain, respectively. These domains are capable of activation independently of each other but both are required for normal *vg* function (MacKay et al., 2003). The SID, (Scalloped interaction domain) mediates VG binding to SD and was identified *in vitro* (Simmonds et al., 1998). The light blue region between AD1 and the SID is a domain that appears to be non-essential. The approximate limits of these domains are indicated above the diagram in terms of respective amino acid residues (MacKay et al., 2003).

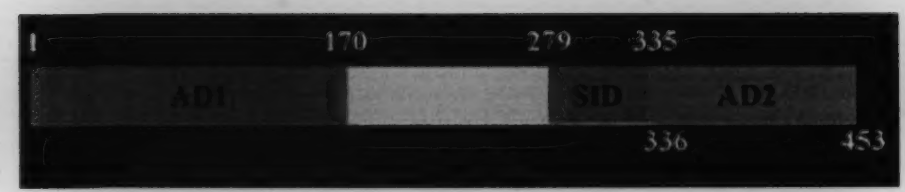

1
170
279
335
AD1
SID
AD2
336
453

Figure 6.2. Control Flp-out GFP clones in the wing disc.

A wing disc induced to produce control Flp-out clones marked by the expression of GFP. The disc is stained with a WG antibody (red). Notice the size and the irregular morphology of the clones. Also, notice that there is no non–autonomous induction of WG by clones overlapping the hinge area. There is no clonal expression of any part of *vg* in this case and so the GFP clones serve as a relevant negative control with respect to the effect of VG function on clone size and morphology.

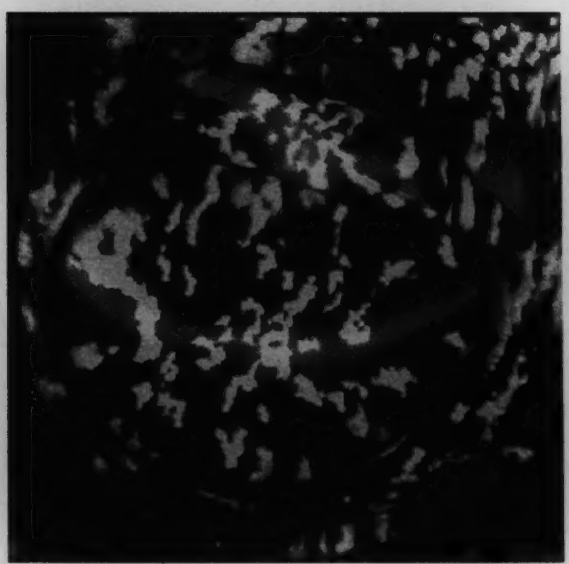


Figure 6.3. Various UAS-*vg* deletion constructs used in this study.

The various *vg* deletion constructs used in this study are shown. The region deleted in each construct is shown by a white rectangle. UAS-*del1-4* deletes the second activation domain (amino acids 356-453), UAS-*del5-6* deletes the first activation domain (amino acids 2-170) and UAS-*del7-8* retains both activation domains but deletes a non-essential region (from amino acids 187-278) in the VG protein. UAS-*vgFull* refers to the full-length *vg*-expressing construct.

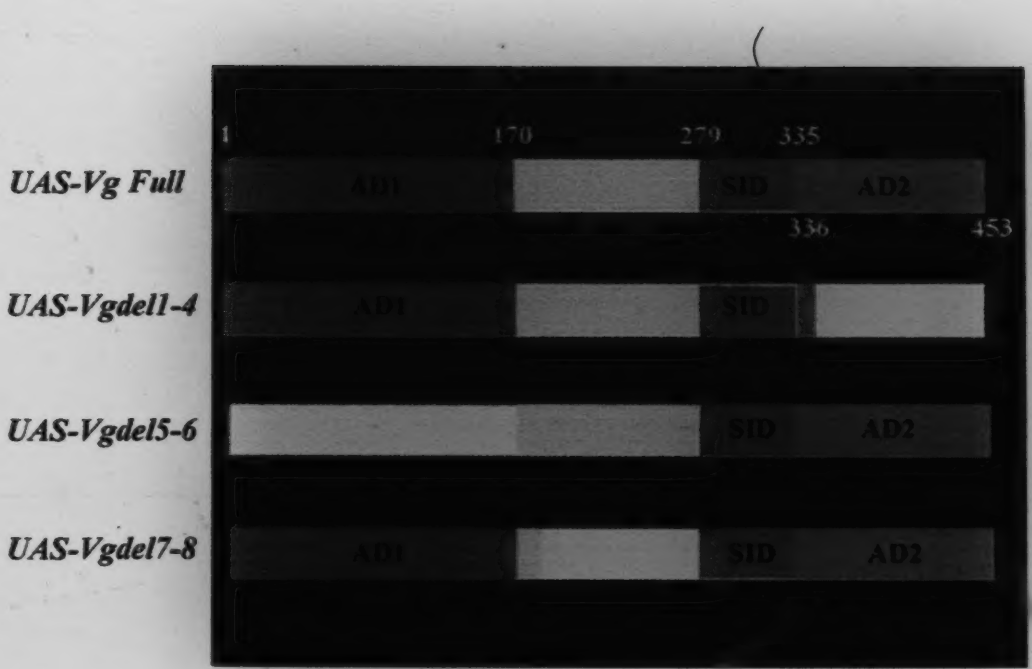

1
170
279
335
336
453
UAS-Vg Full
AD1
SID
AD2
UAS-Vgdel1-4
AD1
SID
UAS-Vgdel5-6
SID
AD2
UAS-Vgdel7-8
AD1
SID
AD2

Figure 6.4. Both activation domains in VG are required for adhesive properties of the cells in which VG is expressed and for the induction of *wg* in the hinge as judged by Flp-out clonal analysis. A-L) Wing discs from late third instar larvae were allowed to grow for between 48-72 hours after clone induction and stained for WG (red channel). The VG- expressing clones are marked by the co-expression of green fluorescent protein (GFP) from an UAS-GFP construct. A-C) Flp-out clones from the construct deleting the 3' putative activation domain of *vg* (UASΔ*vg1-4*). The clones resemble control GFP clones with characteristic irregular shape, small size and inability to induce WG in the hinge region. D-F) Flp-out clones from the construct deleting the 5' putative activation domain of *vg* (UASΔ*vg5-6*). The clones resemble GFP clones as in A-C. G-I) Clones expressing a UASΔ*vg7-8* construct that retains both putative activation domains but deletes an internal 91 amino acid motif. Note that the clones are larger, circular in shape with smooth edges, and now are able to induce WG non-cell autonomously in cells surrounding the clones overlapping the hinge region of the disc. Clones that induce WG are marked by arrowheads. J-L) Flp-out clones from a full-length *vg* construct resemble those in panels G-I.

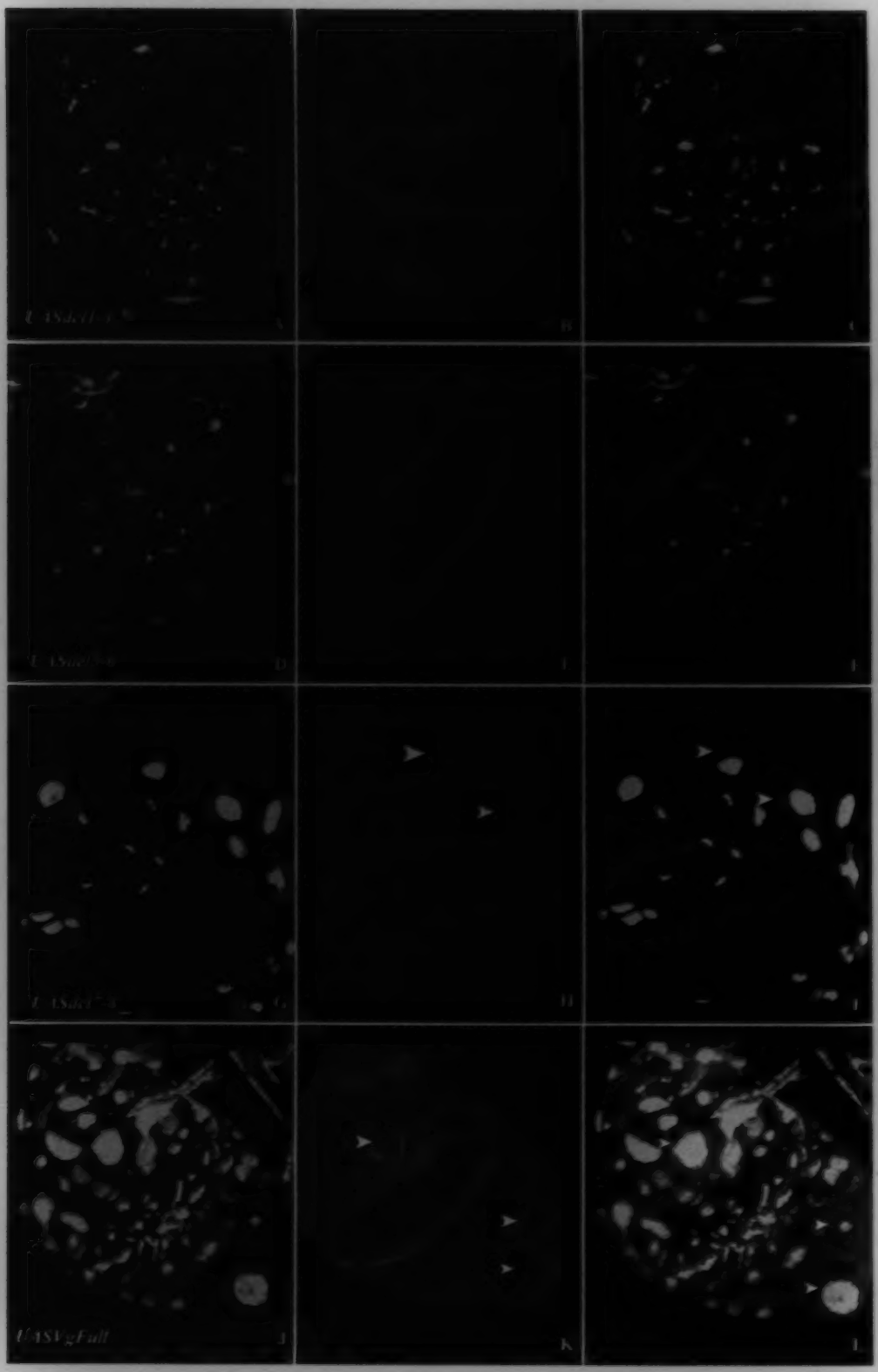
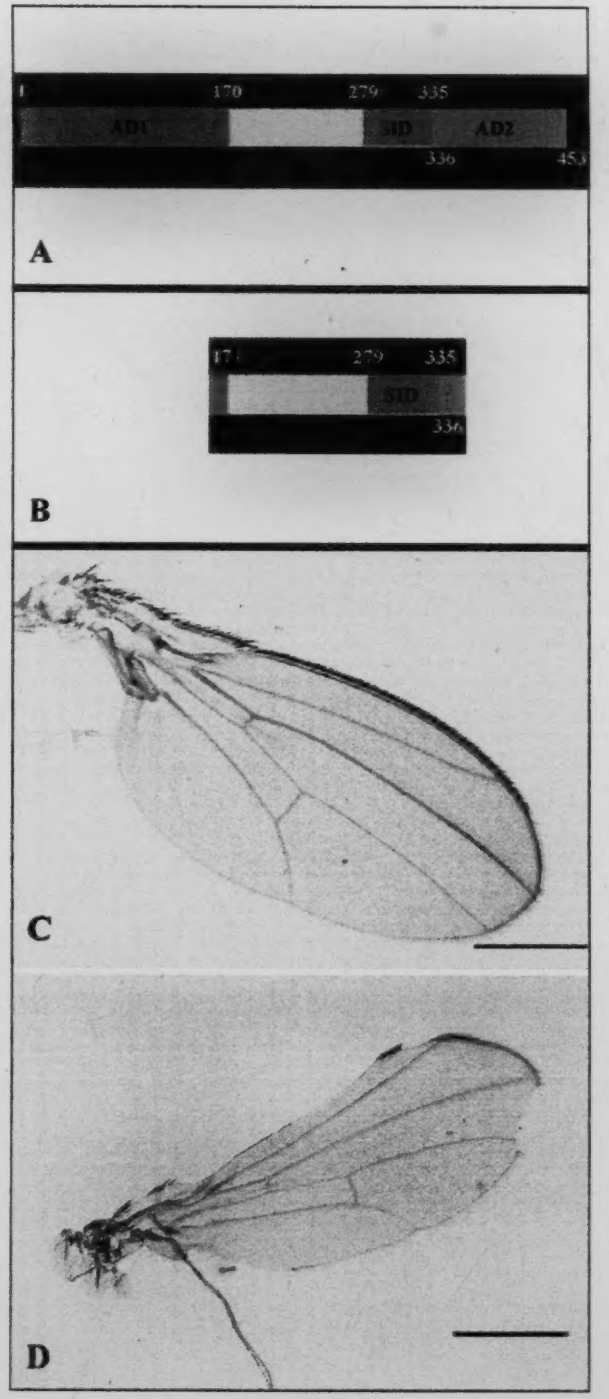


Figure 6.5. *In-vivo* analysis of the Scalloped interaction domain (SID).

A) Domain-structure of VG, portraying the two activation domains and the SID. B) The region of VG spanning amino acids 171-335 found in the UAS-*vgSID* construct. C-D) Whole mounts of adult wings. C) from wild type fly and D) from a fly expressing the VG SID under the control of a *vg*-Gal4 driver. Notice the notching in the wings characteristic of *scalloped* mutations.


1
170
279
335
AD1
SID
AD2
336
453
A
171
279
335
SID
336
B
C
D

Chapter 7

General conclusions and future directions

In this thesis data are presented that help us to further understand the molecular nature of the control of wing development by *vestigial* (*vg*) and *scalloped* (*sd*). The focus is on the role of *sd* in this process, however, some data pertain to the role played by *vg*. In addition, data are presented that link specific amino acid changes in the SD protein to the lethality associated with some *sd* recessive lethal alleles.

Several findings had previously implicated specific roles for *vg* and *sd* in wing development and these findings were the motivating factor behind much of the work presented in this thesis (Halder et al., 1998; Simmonds et al., 1998; Williams et al., 1993). Firstly, mutations in both *sd* and *vg* were shown to have a similar wing phenotype (associated with notching in the wing). Secondly, both genes were shown to be expressed in similar patterns within the wing pouch. Thirdly, the encoded proteins had been shown to bind to each other *in vitro*. Based on the above findings, it was postulated that the *sd* and *vg* gene products co-operatively functioned as a transcription complex wherein *sd* provided the DNA binding ability and *vg* provided the activation function. While there was some genetic evidence that gave credence to the above hypothesis it had not definitively been shown that, indeed, this was the case. It was also known that VG (which lacks a nuclear localization signal) binding to SD was important for its nuclear localization (Halder et al., 1998; Simmonds et al., 1998). Because the SD TEA domain has a putative bipartite NLS, I hypothesized that VG was getting translocated into the nucleus via this NLS, by virtue of its ability to bind SD.

In chapter two, the problems mentioned above are addressed and data are presented that complement previous studies and conclusively show that SD and VG act as a transcription complex wherein SD provides the DNA binding ability via the TEA domain and VG provides the activation function. It is also shown that the SD TEA domain is responsible for translocating SD into the nucleus via the NLS found within the TEA domain, and thus accounts for the transport of VG into the nucleus as well. This is shown by constructing and characterizing the function of a fusion between full length VG and the TEA domain of SD spanning amino acids 63-211. The reasoning behind constructing the fusion was as follows. If, in the SD/VG complex, SD provided the DNA binding ability and VG the activation function then it was possible that a fusion between VG and the SD TEA domain could mimic the native transcription complex and thus could rescue a *sd* wing mutation. In fact, the fusion is able to rescue a *sd* wing mutation as well as the phenotype of a *sd, vg* double mutant, thus conclusively demonstrating that the fusion functions as the native transcription complex. This also demonstrates that, within the context of wing development, the SD TEA domain is critical and the rest of the protein, in principle, is non-essential. Over-expression of VG in wing discs from a *sd* mutant background leads to a cytoplasmic localization of VG. However, expression of the fusion in the same *sd* background results in nuclear localization of VG, thus demonstrating that the NLS contained within the TEA domain of SD is likely responsible for the nuclear localization of SD and, indirectly, VG. A further refinement of the 63-211 amino acid region of SD critical for wing development and nuclear localization of VG was also successful. The critical area can now be said to reside within amino acids 87-185 and data in support of this are presented in chapter four.

In chapter three data are presented that provide a plausible molecular explanation for the rescue of *sd* mutations by the fusion construct. It was previously shown by clonal analysis that *sd* mutations are deficient in expression of *wg* (Liu et al., 2000; Varadarajan and VijayRaghavan, 1999). It is shown that *sd* mutants are deficient in the expression of the dorso-ventral stripe of *wg* in the wing pouch and rescue of this stripe by the fusion construct results in rescue of the *sd* wing mutation that is accompanied by cell proliferation. Furthermore, it is demonstrated that, while expression of exogenous *wg* can also rescue the *sd* wing mutation, expression of a dominant negative form of TCF cannot and results in a *sd* wing phenotype. dTCF is a known transducer of the *wg* signal (van de Wetering et al., 1997) and a defect in this transducer results in decreased WG signal. It is also shown that the fusion construct is able to induce expression of a proneural gene *senseless* (*sens*) (Nolo et al., 2001) that is responsible for bristle morphogenesis at the wing margin. Based on these data, a model is presented that describes the relationship between *vg/sd*, *wg* and *sens* during wing development. It is postulated that *wg* expression at the boundary is maintained or induced by the SD/VG complex. It is also proposed that *sd* function is needed at the margin for the development of SOPs by *sens*.

Apart from its role in wing development, *sd* also has a fundamental role in the viability of the fly (Campbell et al., 1991) and in chapter five data are presented from the molecular characterization of several *sd* recessive lethal alleles. When these lethal alleles are homozygous, lethality ensues in either early larval or late pupal stages. Furthermore, four of these alleles do not complement the wing phenotype of a hypomorphic *sd* allele whereas one does complement. The data presented from the characterization of these alleles led to a model proposing to explain the observed heterogeneity of heterozygous

wing phenotypes exhibited by the lethal alleles in the non-complementing combinations. Three of the wing non-complementing alleles (sd^{3L}, sd^{47M}, sd^{EM68}) have mutations that affect a putative VG interaction domain (Maeda et al., 2002; Vaudin et al., 1999) and thus help define the limits of this interaction domain. Because this interaction between SD and VG is important for the formation of a functional SD/VG complex, lesions within this domain result in a lowering of the amount of functional complex and hence the wing non-complementing phenotype in trans with hypomorphic *sd* alleles. One of the wing non-complementing alleles (sd^{31H}) affects the third helix of the TEA domain and could prevent binding of SD to DNA (Burglin, 1991). This could result in lower numbers of functional SD/VG complexes, manifesting in a wing phenotype in trans with a hypomorphic *sd* allele. The sd^{11L} lethal allele complements the wing phenotype of weak alleles of *sd* and the mutation in this lethal allele doesn't affect the VG binding domain. Thus, normal levels of functional SD/VG complexes are likely produced and this results in complementation of the wing phenotype. Further support for this comes in the form of the defective VG localization in wing discs from hemizygous males from sd^{EM68} and sd^{47M} alleles. In these cases, VG is diffuse and predominantly cytoplasmic. However, in discs from sd^{11L} larvae, VG localization is nuclear, indirectly suggesting that indeed the wing non-complementing alleles affect the binding of VG to SD. Because this binding is necessary for the nuclear localization of VG, a disruption results in cytoplasmic localization of VG. Because these alleles are recessive lethals and the lesions fall within the C-terminal half (except for sd^{31H}), it may be that the lesions also affect the binding of other factors to SD that would activate transcription of genes important for the viability of the fly.

One can propose that the C-terminal half of SD is possibly used to bind transcription intermediary factors (TIFs) specific to different tissues to activate transcription at different stages of development. Thus in the wing, SD binds to its TIF VG. Putative TIFs from other tissues where *sd* is expressed have yet to be identified.

It has been proposed (Liu et al., 2000) that the organization of the wing is dependent upon an interaction between *vg* expressing wing pouch cells and the hinge cells. They show that clones over-expressing *vg* are capable of inducing *wg* at the hinge in a non-cell autonomous fashion. This result has also been recently demonstrated in a report that looks at the role of *vg* in organization of the wing hinge (Rodriguez D del et al., 2002). Liu et al., (2000) also show that *vg* regulates adhesive properties of the cells that express it and this possibly leads to separation of wing hinge from the pouch. Furthermore, VG has been demonstrated to have two activation domains *in vivo* (Mackay et al., 2003). In chapter six the role of these activation domains is explained with respect to induction of *wg* in the hinge and regulation of adhesive properties. The data presented in that chapter, show that for induction of *wg* and adhesion of cells both activation domains must be present. Either of them alone is insufficient to carry out the above two functions. The *in vivo* effects of expressing the SD interaction domain (Simmonds et al., 1998) were also examined and the data demonstrate that this domain, when expressed in the wing, functions just as predicted *in vitro*. That is, this construct can impart a dominant negative effect in otherwise wild type flies. This is consistent with the role of this domain in binding SD. This binding possibly results in a lower number of functional SD/VG complexes resulting in the wing phenotype, because a construct containing only a SID would act as a competitive inhibitor to full length VG binding. Thus, the construct

carrying this domain could be used to titrate SD from specific tissues by directing its expression ectopically. This would be helpful in understanding the role that *sd* might be playing in tissues other than wing during development.

With respect to *sd*, the data presented in this thesis advance our understanding of the function of *sd* in several important ways.

1) A region from amino acids 87-185 in SD is critical for wing development and the rest of the protein is dispensable in this context. This stretch of the protein contains the TEA DNA-binding domain as well as the NLS for SD. Also, the NLS contained within the TEA domain of SD is responsible for the nuclear localization of VG, by virtue of the ability of VG to bind to SD in a domain separate from the TEA DNA binding domain.

2) Hypomorphic but viable *sd* wing mutations can be rescued by *wg*, suggesting that *sd* is upstream of *wg* in the genetic hierarchy of wing development.

3) *sd* function is likely needed for specification of SOPs by *sens*.

4) The C-terminal end of SD contains a VG interaction domain that is compromised in several *sd* lethal mutations studied.

5) The lesion in sd^{31H} lethal allele is in the TEA DNA binding domain and results in mutant wing and a recessive lethal phenotype.

6) A model to explain the molecular basis for the phenotypes observed with various lethal alleles is presented.

In addition, the data in this thesis also further our understanding of VG function in the context of its partnership with SD.

1) The VG protein provides the activation function in the SD/VG complex. For normal activation both activation domains must be present.

2) The SID from VG can bind SD *in vivo* on its own. This causes a dominant negative wing phenotype because this binding will competitively inhibit the ability of full length endogenous VG to bind to SD.

While this thesis furthers our understanding of *sd* and *vg* during wing development many questions still exist that will be addressed in future studies, and several of these are listed below.

1) It has been proposed that the binding of VG to SD changes the binding selectivity of the SD TEA domain to certain specific sites. It has also been proposed that this could be a way of limiting the number of functional sites for this complex in the genome (Halder and Carroll, 2001). This was based entirely on *in vitro* experiments and did not take into account the post-translational modifications that could occur within the TEA domain that have been reported from other organisms (Gupta et al., 2000; Jiang et al., 2001; Jiang et al., 2000). In the report by Halder et al. (2001) it was also proposed that for the change in binding selectivity of the TEA domain, regions outside of the SID in VG were important. However, it has been shown that a human homologue of *vg*, TONDU, that has weak homology within the SID and none outside of this domain, is capable of partially rescuing *vg* mutations (Vaudin et al., 1999). If, indeed, regions outside of the SID were important for a change in target selectivity by the TEA domain then TONDU should not be able to rescue the *vg* mutations, as TONDU has no homology outside of the SID. Since TONDU can partially rescue *vg* wing mutation this suggests that for target selectivity something else is needed. Recent studies from mammalian systems have shown that phosphorylation of the TEA domain could modify the ability of the TEA domain to bind to DNA (Gupta et al., 2000; Jiang et al., 2001). To test if target selectivity

of the TEA domain is VG- independent, *in vivo*, a fusion was made between the generic activation domains from the HSV VP16 and the TEA domain from SD. This construct has been injected in the flies and the process of establishing transgenic lines is in progress. These transgenic flies would be employed in experiments that would test the ability of this construct to rescue sd^{58} wing mutations. A wing rescue from such an experiment would mean that VG might not have a role in target selectivity by TEA domain *in vivo*. Further, it could imply that the target selectivity possibly comes about by mechanisms other than VG binding to SD, possibly by tissue specific post-translational mechanisms like phosphorylation.

2) Characterization of suppressors of *sd* wing phenotype: In an EMS screen for suppressors of sd^{58} wing phenotype, three suppressors have been identified that show significant suppression of the wing phenotype. These remain uncharacterized simply due to a lack of time on my part. The first step in the characterization would be to map the suppressors to the autosomes and then carry out further characterization in terms of the genes that these suppressors affect. Understanding the genes that these suppressors affect could help us further elucidate the genetic circuitry important for wing development.

3) Identification of targets of *sd* by microarray analysis: Identification of genes that are controlled by *sd* would be another way of understanding what processes during development are regulated by *sd*. The existence of lethal alleles of *sd* offer an opportunity to identify targets of *sd*. In a preliminary experiment I have identified ~500 genes that are either up or down regulated between RNA pools from wild type and *sd* mutant animals. The experiment needs to be repeated to verify if these genes are indeed affected by

mutation in *sd* and further characterization of these genes would help us understand their role in development.

4) Does the *sd* locus undergo alternative splicing? It has been suggested that the *sd* transcript undergoes alternative splicing to produce multiple mRNAs. Data in support of this come in the form of full-length cDNAs that appear to differ significantly. At least three of these cDNAs have been reported and called E21, E16, E7 (Campbell et al., 1991). While E21 and E16 are similar except in their UTRs, E21 and E7 differ significantly in the protein that they encode. The E7 isoform appears to code for a protein that would produce part of the TEA domain and then differ beyond this point. So far no mutants have been recovered that would affect the alternative isoforms and it is possible that these cDNAs may merely be experimental artifacts. An experiment outlined below could help in determining whether the cDNAs reported are due to alternative splicing or not. The experiment would involve generating transformants carrying one of two specific constructs. Both constructs would be fusions between either *lacZ* or GFP and genomic regions from the *sd* locus. The genomic region included would differ in the exon intron region so that splicing would generate a product specific to either the E21 or E7 isoform. It is possible that this splicing could be occuring in a tissue specific manner. Fusing the genomic region (including specific introns and exons) with a GFP or a LacZ at the C-terminal would allow one to differentiate between an E21 or E7 form of splicing. If, indeed, alternative splicing is responsible for producing the above mentioned cDNAs then the different GFP or LacZ fusions would be helpful in reporting any tissue specific expression. A functional GFP or LacZ protein would be produced, only if a particular type of splicing occurs and results in an in-frame fusion between the alternatively spliced

genomic region with the GFP or LacZ. The knowledge gained could help settle the issue of whether the *sd* locus undergoes alternative splicing or not.

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